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REGULATION OF METHYLTETRAHYDROFOLATE SYNTHESIS IN AERATED CARROT TISSUE DISKS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled 'Regulation of Methyltetrahydrofolate Synthesis in Aerated Carrot Tissue Disks' submitted by Paul Fedec in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Biochemistry.

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ABSTRACT

A detailed examination of tetrahydrofolate derivatives, their synthesis and turnover in aerated carrot tissue disks has been conducted. Derivatives were assayed microbiologically with *Lactobacillus casei* (ATCC 7469) and *Streptococcus faecalis* (ATCC 8043) after separation by DEAE-cellulose chromatography and treatment with γ-glutamyl carboxypeptidase. At initial slicing, carrot tissues contained 17-42 ng folic acid equivalents/g fresh wt. as assayed by *L. casei*. The major derivative found was 5-methyltetrahydrofolate, representing 61% of the free folates assayed. Lesser amounts of 10-formyltetrahydrofolate mono and diglutamate forms were also detected. Quantities of these derivatives increased substantially upon aeration of carrot slices in distilled water, 0.1 mM gibberellic acid, 5.0 mM L-methionine and 10.0 mM L-ethionine. Largest increases in the 5-methyl derivative were found in methionine-treated slices. A substantial portion of the folate pool was found to exist as methyl polyglutamyl derivatives.

The activity of enzymes participating in folate-mediated C_1 transfer reactions was studied *in vivo* and *in vitro*. 10-Formyltetrahydrofolate synthetase, 5,10-methylenetetrahydrofolate reductase, 5-methyltetrahydrofolate:homocysteine transmethylase and serine hydroxymethyltransferase showed considerable increases in specific activity during aeration of carrot slices in distilled water, gibberellic acid, L-methionine and L-ethionine. Repression of these enzymes by L-methionine was not apparent. Within specific concentration ranges, L-methionine showed some inhibition of the first three enzymes mentioned.

Incorporation of ^{14}C from [^{14}C]formate was followed in pre-treated carrot tissue disks. Slices aerated in distilled water, gibberellic acid and L-methionine, in comparison to freshly-cut slices, showed enhanced ability to incorporate ^{14}C into CO_2, lipids, sugars, organic and amino acids. When the specific radioactivity of free and protein amino acids was determined, it was apparent that aerated slices from all three treatments had greater ability to incorporate formate into free amino acids related to one-carbon metabolism such as serine, methionine and glycine. Methionine had the highest specific activity in all three treatments.

A study of free amino acids in carrot tissue revealed that this pool changed drastically with length of storage of the mature roots. Longer stored tissue showed elevated free amino acid levels. During aeration of slices in distilled water, gibberellic acid or methionine, the free amino acid pools declined dramatically. On the other hand, protein amino acid levels increased during the later stages of aeration.

Respiration rates were found to increase more in carrot slices aerated in gibberellic acid and 0.02 mM aminopterin than in L-methionine which gave values similar to those of the distilled water control. Respiration in carrot disks was inhibited by L-ethionine.

It is concluded that tetrahydrofolate derivatives play a significant role in the enhanced biosynthetic processes known to occur in storage tissue slices undergoing aeration.

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LIST OF ABBREVIATIONS[†]

C_1	:	carbon-one
THFA[††]	:	tetrahydrofolic acid
5-CH_3-THFA	:	N^5-methyltetrahydrofolic acid
10-HCO-THFA	:	N^{10}-formyltetrahydrofolic acid
met	:	methionine
SAM	:	S-adenosyl-L-methionine
ATCC	:	American Type Culture Collection
EC	:	Enzyme Commission
g, µg, ng	:	gram, microgram, nanogram
M	:	molar
µmol, nmol	:	micromole, nanomole
EMP	:	Embden-Meyerhof pathway
GSH	:	reduced gluthathione
Tris	:	tris(hydroxymethyl)aminomethane
IAA	:	indoleacetic acid
GA_3	:	gibberellic acid A_3
conc.	:	concentration
ADP, ATP	:	adenosine diphosphate, adenosine triphosphate
NADP	:	nicotinamide-adenine dinucleotide phosphate

[†] Other abbreviations commonly utilized in the text are given in the format acceptable for publication in the *Biochemical Journal* as listed in vol. 126:1-19 (1972).

[††] Although it is recognized that tetrahydrofolic acid and its derivatives have synonymous names, for example, tetrahydropteroylglutamic acid (H_4PteGlu), as suggested by the IUPAC-IUB Commission (*Biochem. J.* 102:15 (1967)); for the sake of continuity between common usage in the literature, enzyme nomenclature and this text, THFA will be used.

LIST OF ABBREVIATIONS (cont.)

µm	:	micron
µCi	:	microCurie
FAD	:	flavin-adenine dinucleotide
GDP, GTP	:	guanosine 5'-pyrophosphate, guanosine 5'-triphosphate
DEAE-cellulose	:	diethylaminoethyl-cellulose
mRNA, tRNA	:	messenger RNA, transfer RNA
c.p.m.	:	counts per minute

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INTRODUCTION

Studies during the past two decades have revealed that higher-plant storage tissues undergo considerable metabolic transformation following slicing and 'aging' of such tissues at physiological temperatures. These induced metabolic activities include: activation of a large number of enzymes; synthesis of new protein, RNA and DNA; an increased ability to metabolize exogenous substrates; differential response to application of growth-regulating substances and a marked increase in respiration rate.

The enhancement of such processes directly or indirectly implicate one-carbon metabolism playing a significant role in the biosynthesis of key intermediates required for such activities as those mentioned earlier. To date, absolutely no investigation of any kind has been directed at elucidating the interrelationship of biochemical events occurring in aerated storage tissue disks to a dependence on C_1 metabolism.

In keeping with the current research of this laboratory, the production and regulation of one-carbon units were the basic objectives of this study. It was proposed, then, to utilize aerated storage tissue disks as a dynamic system which afforded not only ease of handling and some degree of homogeniety of higher-plant tissue but would also exhibit active biosynthesis of methyl groups. To expand the scope of the study, use of gibberellic acid, by virtue of its ability to enhance methylation and nucleic acid synthesis, was introduced as a means of manipulating the metabolic activities of this system in an effort to facilitate investigation of the role folate derivatives and related enzymes play during aeration.

To bring the study into perspective, it is proposed to review literature having direct bearing on the present investigation in both fields with only brief reference to other basic research. On the one hand, an attempt will be made to orientate the reader to the 'system' and the dynamic changes that occur during aeration which implicate involvement of C_1 metabolism. On the other hand, a review of one-carbon metabolism will be made, its occurrence and regulation in plants, micro-organisms and animals, as well as the interrelationship the two major areas have to the present investigation.

LITERATURE REVIEW

I. Aeration or Aging of Storage Tissue Disks

Extensive use for purposes of physiological and biochemical experimentation has been made of disks cut from storage tissue organs of various plant species. Since the present investigation dealt entirely with one specific tissue, namely carrot (*Daucus carota* L.), the literature dealing with pertinent features of such a system will be reviewed from the standpoint of carrot first, supplemented by information drawn from other tissues for areas not studied in carrot.

A. Definition of Aeration as Opposed to Aging

Thin slices of disks, ranging from 0.3 mm to 20.0 mm in diameter and 0.2 mm to 2.0 mm in thickness, have been cut from cores of storage tissues such as carrot, potato (*Solanum tuberosum* L.), Jerusalem artichoke (*Helianthus tuberosus* L.), beetroot (*Beta vulgaris* L.), sugar beet (*B. vulgaris* var. *saccharifera*), turnip (*Brassica rapa* L.) and sweet potato (*Ipomoea batatas* Lam.) and placed into various solutions, distilled water being the most common, through which air is circulated causing a washing, floatating or circulating action. This process, continued for short periods of time, is generally termed 'aging' in the literature. It has also, on occasions, been referred to as 'incubation' or 'aeration'. The term 'aging' is probably more appropriate when used in reference to tissue which is undergoing senescence rather than tissue undergoing enhanced biosynthesis, as is the case in aerated sliced storage tissue disks. This thesis will, therefore, use the term 'aeration' to refer to the process

wherein sliced disks are placed in a liquid medium through which air is passed for defined periods of time.

B. Bacterial Contamination of Disks during Aeration

In early observations of metabolic changes occurring in aerated disks little attention was paid to conditions of aeration or the medium used. For example, MacDonald *et al.* (1961) used tap water as a medium for disks in some of the original studies. It was not until recently that Leaver and Edelman (1965*b*) recognized the fact that artichoke and carrot slices became contaminated with relatively large amounts of bacteria. In some cases it was shown that as many as 10^2 bacteria were initially present per carrot disk which increased to 5×10^5 bacteria per disk after 24 hr of aeration in distilled water. These bacteria were all of the pseudomonad type (Edelman and Hall, 1965). Clearly, such numbers of bacteria could significantly alter or contribute to biochemical processes in the higher plant system and could erroneously contribute to the parameters being observed. Bacterial numbers can be effectively reduced by use of 50-100 µg/ml chloramphenicol (Leaver and Edelman, 1965*b*; which reduced bacterial numbers to several hundred per carrot disk after three days of aeration), 5.0 mM $CaCl_2$ (Wildes and Neales, 1971) or simply by employing aseptic techniques along with frequent changes of the medium (Edelman and Hall, 1965; Reed and Kolattukudy, 1966). The latter approach resulted in artichoke and beetroot slices which were essentially free of bacteria after two days of aeration or at worst contained only low numbers per disk.

C. Observable Metabolic Changes

1. Respiration

One of the parameters first observed in aerated storage tissue slices was that of increased respiration rate. Slicing of such tissues followed by aeration generally led to a marked increase in respiration (Steward *et al.*, 1940; Laties, 1960; Ap Rees, 1966; Davies, 1966). This increase, commonly termed 'induced respiration', is dependent for its initiation upon slicing, upon aerobic metabolism of the slices and is characteristically accompanied by marked changes in metabolism to be reviewed in appropriate sections.

Following placement of freshly cut carrot disks in aerated distilled water, there is an immediate decline in respiration rate as measured by oxygen consumption (Adams, 1970*a*). After a recovery period of about two hours, two distinct stages of rate increase are observed, the first extending to 24 hr and the second after 40 to 60 hr of aeration (Adams and Rowan, 1970). The respiration rates have usually been observed to approximately double within 24 hr in such systems (Steward and Preston, 1940; Adams and Rowan, 1970).

Respiration rates in assorted other storage tissue also show substantial increases upon slicing and aeration. Early work by Steward *et al.* (1940) indicated that potato tuber slices placed in salt solutions showed large increases in respiration in response to aeration. Laties (1960) showed that increases in the respiration of potato slices in distilled water were inversely proportional to slice thickness. Slices 0.5 mm thick showed a 400% increase in respiration over a 24 hr period.

Plausible reasons for this increase in respiration were originally proposed by Laties (1959). It was believed that the respiratory rise was initiated by removal of a restraint on phosphorylation activity of the tissue. This was later modified to include an activation of pre-existing mitochondria (Fowke and Setterfield, 1968). More recently it has been shown that induced respiration is dependent upon synthesis of RNA and protein (Ap Rees and Royston, 1971). Such renewed synthetic activity could lead to development of induced respiration in two ways (Ap Rees and Royston, 1971). First, increased demands for ATP could increase the activity of respiratory enzymes present in the fresh tissue slices and second, there may be a net synthesis of enzymes related to respiration as exemplified by the rise in invertase. Ap Rees and Royston (1971) concluded that increased respiration must be the result of increased synthesis caused by slicing and aeration of disks rather than the reverse (Laties, 1963).

2. Changes in sugars, organic, amino and nucleic acids during aeration of storage tissue disks

Alabran and Mabrouk (1973) reported that free sugars make up 8.2% of the fresh carrot and constitute 91% of the total sugars. Of this total; sucrose, β-glucose, α-glucose and fructose make up the majority, 37.9%, 20.8%, 16.2% and 11.7%, respectively. During aeration of carrot slices, the main metabolic route of supplied glucose indicates that it is converted to sucrose and then oxidized via the EMP and pentose-phosphate pathways (Ap Rees and Beevers, 1960).

During growth, carrots show a regular accumulation of soluble sugars with sucrose being the principal sugar present at the time of maturity (Platenius, 1934). Upon storage this sugar composition changes

(Goris, 1969) with the levels of sucrose nearly doubling after three months of storage while those of glucose and fructose decrease by fifty percent. Free sugars have been shown to decline after slicing and aeration of these tissues (Ap Rees and Beevers, 1960).

Splittstoesser (1966) detected malate during [^{14}C]leucine incorporation studies in carrot tissue. Otsuka and Take (1969) detected relatively low concentrations of α-ketoglutarate, succinate, glycolate, lactate and citrate in fresh carrot root tissue while Lacharme and Nétien (1969) noted that aconitate and isocitrate were also present, however, the latter were carrot tissue cultures. MacDonald *et al.* (1961) noted that during the first 72 hr of aeration of beetroot slices there was an overall increase in most organic acids and as much as a 300% increase in a few individual acids.

The amino acid composition of carrot tissues has been examined by several workers (Bourke *et al.*, 1967; Otsuka and Take, 1969; Alabran and Mabrouk, 1973). According to Alabran and Mabrouk (1973), the principal amino acids are aspartate, serine, glutamate and alanine. In contrast, Otsuka and Take (1969) found little aspartate in their analysis but noted substantial quantities of threonine and valine. Bourke *et al.* (1967) also found substantial amounts of threonine and noted the total free amino acid content of carrot, purchased from a local market, to be about 3.73 µmol/g fresh wt.

Little is known about changes in amino acid composition in carrot during aeration, however, in beetroot the total amino acid content, estimated as soluble nitrogen, falls during aeration of slices (MacDonald

et al., 1961). Thurman *et al.* (1963) also noted that most amino acids declined during aeration of beetroot disks with the exception of glutamate. Their studies revealed that the most common amino acids of beet disks were similar to those of carrot (Alabran and Mabrouk, 1973); namely, aspartate, glutamate, serine, glutamine, asparagine and threonine. Little methionine was found in beet tissue (Thurman *et al.*, 1963).

If such tissues, low in methionine, are able to support active protein synthesis and RNA methylation during aeration, clearly turnover of methionine must be very important. This turnover, in relation to metabolic changes during aeration of storage tissue disks, is open to investigation.

During induced respiration in carrot disks, increases in RNA content of approximately 50% have been reported after aeration for 24 hr (Leaver and Edelman, 1965*a*). Polyribosome formation is enhanced during the first 6 hours of aeration (Leaver and Key, 1967; Lin *et al.*, 1973) and is believed to be one of the earliest biochemical changes associated with aeration of storage tissue disks. Such a change is believed to underlie the onset of protein synthesis and is probably dependent on mRNA synthesis, the latter being essential for polyribosome formation (Leaver and Key, 1967).

In addition to RNA synthesis, Bryant and Ap Rees (1971) have also noted that net synthesis of DNA occurs in carrot slices aerated for 48 hr. They concluded that induced respiration was dependent upon the synthesis of new RNA and protein.

In other tissues subjected to slicing and aeration, increases in RNA synthesis have also been noted (e.g., Ellis and MacDonald, 1967; Vaughan and MacDonald, 1967; Sampson and Laties, 1968; Rose *et al.*, 1972). In beetroot and potato slices, this has largely been rRNA (Ellis and MacDonald, 1967; Sampson and Laties, 1968). As in carrot, it appears that induced respiration in potato disks is dependent upon RNA and protein synthesis (Brinkman *et al.*, 1973). Such increases in nucleic acid synthesis are believed to stem from the slicing of tissue into disks inducing a derepression of gene activity which subsequently is responsible for the synthesis of both ribosomal and messenger RNA (Ellis and MacDonald, 1967; Vaughan and MacDonald, 1967). The synthesis of new mRNA then leads to activated protein synthesis (Ellis and MacDonald, 1968).

Synthesis of nucleic acids requires one-carbon units that must either be derived from an available folate pool or be actively synthesized as needed. The involvement and control of C_1 metabolism as related to enhanced nucleic acid biosynthesis induced by aeration of tissue slices is an area to which investigation has not been directed.

3. Enzyme activity and protein synthesis

Until recently, little information was available regarding changes in enzyme activity and protein content in carrot tissues although MacDonald *et al.* (1961) demonstrated a net synthesis of protein in aerated beet disks. These early measurements were made on the basis of changing nitrogen contents of the tissues. More recently, however, measurement of protein synthesis has been in terms of enzyme synthesis with invertase being a common parameter.

Ricardo and Ap Rees (1970) noted that increases in invertase activity in aerated carrot slices were accompanied by a decline in sucrose levels. In common with Jerusalem artichoke, carrot disks appeared to produce a proteinaceous substance capable of inhibiting the development of invertase in freshly cut tissues (Bradshaw *et al.*, 1970). Enzymes of the pentose phosphate pathway, particularly 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase increase within 24 hr of aeration in carrot slices (Ricardo and Ap Rees, 1972).

In a comprehensive study of enzyme activity in aerated potato slices, Sacher *et al.* (1972) observed that, of 17 enzymes studied, most were synthesized *de novo* during aeration. The most significant increases occurred in phenyl-ammonia lyase, shikimate dehydrogenase and ribonuclease.

Jerusalem artichoke slices have also been examined for activation of enzymes during aeration. For example, increases in invertase and ascorbate-oxidase activities have been clearly observed (Edelman and Hall, 1965) and rapid increase in the levels of peroxidase, catalase, cytochrome oxidase and phenol oxidase have been noted (Bastin and Dijkmans, 1970). These increases are thought to be due to a stimulation of the rate of enzyme synthesis rather than a decrease in the rate of decomposition (Bastin and Ünlüer, 1972).

Sacher *et al.* (1972) have proposed a plausible explanation for the widespread yet often selective increase in enzyme activity noted during aeration. They believe that the general increase in protein synthesis could be due to derepression allowing production of a wide range of messenger RNAs. The selectivity in mRNA production could explain why some enzymes fail to show an increase during aeration.

4. Structural and morphological changes

Various reports indicate that growth and/or cell differentiation may occur after slicing. Phloem slices of carrot tissue less than 0.5 mm in thickness were found to have vessel element formation after eight to nine days of aeration but slices greater than 0.55 mm thick showed no formation of vessel elements even after one month (Mizuno *et al.*, 1971).

Studies of Jerusalem artichoke have shown that the number of cells per disk did not change during an aeration period of two days (Edelman and Hall, 1965). Before aeration such tissues have an elementary endoplasmic reticulum and small nucleoli but following aeration an increase in endoplasmic reticulum and attached ribosomes occurs (Fowke and Setterfield, 1968).

After aeration, potato slices showed a marked increase in mitochondrial numbers (Verleur, 1969) and these mitochondria tended to be a heterogeneous population having different sedimentation velocities, complexity and biochemical properties (Verleur *et al.*, 1970). In potato mitochondrial preparations, the rate of oxygen uptake was limited by ADP levels, substrate oxidation being immediately accelerated on addition of ADP *in vitro* (Verleur, 1965).

D. Effects of Growth Regulators and Other Exogenous Treatments on Metabolism of Aerated Disks

In aerated beetroot slices (Palmer, 1966), GA_3 concentrations of 10^{-4} to 10^{-6} M enhanced respiration, absorption of phosphate and invertase activity. IAA at 10^{-3} M inhibited the above parameters. In later work, Palmer (1970) noted that in artichoke slices phosphatase

activity did not increase with aeration but on addition of 10^{-4} M IAA a 300% increase was observed. No such stimulation occurred when GA_3 and kinetin were added. Invertase synthesis in artichoke slices was shown to increase as a result of GA_3 application but physiological concentrations of IAA inhibited invertase synthesis (Edelman and Hall, 1964). In the same study, these workers found the synthesis of invertase in carrot disks was suppressed by IAA.

Not only do growth regulating substances have a profound effect on enzyme activity of storage tissues, it has been noted that when used alone or in combination they are capable of inducing growth or differentiation of such systems. It is generally accepted that GA_3, when used alone, is not capable of promoting the growth of artichoke cells but auxins and kinetin may act synergistically to promote either cell division or cell expansion (Setterfield, 1963; Fowke and Setterfield, 1968). Auxin, applied after an aeration period, induces true growth as evidenced by increases in structural protein, organelles and general cell expansion (Fowke and Setterfield, 1968). Yeoman and Mitchell (1970) found that during aeration of artichoke tuber slices, the age of the tuber and its post-harvest treatment were important in determining whether cells subjected to aeration would show expansion or division.

Explanation of the mechanism behind the effects of many growth regulating substances is still the subject of research. Fowke and Setterfield (1968) determined that auxin was not essential for RNA synthesis in artichoke slices, and that GA_3 suppressed cell division. Using this information, Kamisaka and Masuda (1970) found that supplying GA_3 to aerated artichoke slices stimulated the incorporation of [^{14}C]thymidine into the

DNA fraction and therefore concluded that GA_3 stimulates DNA synthesis not related to cell division. Bamberger (1971) attempted to further explain the action of hormones on the basis that since GA_3 and IAA are initiators of RNA synthesis because of their capacity to loosen both the binding of histone to DNA and DNA to DNA in the double helix; then GA_3 must be responsible for increasing the number of template sites on DNA. Synthesis of a small but specific RNA fraction would then follow. This specific RNA fraction might then be responsible for increased synthesis of specific enzymes as in the case of α-amylase and ribonuclease in barley aleurone following GA_3 treatment (Chrispeels and Varner, 1967).

These findings raise the possibility that GA_3 treatment of tissue disks may enhance protein synthesis via stimulation of nucleic acid biosynthesis. Whether the additional C_1 groups are already available or have to be synthesized *de novo* is of interest.

Exogenous amino acids have also been added to the liquid medium in an effort to establish the nature and regulation of biosynthetic pathways in aerated storage tissue disks. L-methionine (10^{-3} M) and its analogue L-ethionine have been found to inhibit respiration in aerated turnip disks (Davies, 1966). As S-adenosyl methionine (SAM) accumulated in turnip disks when L-methionine was supplied, Davies proposed that the latter amino acid inhibited respiration by trapping adenosine and thereby limiting the rate of oxidative phosphorylation. Similar data were obtained for aerated sugar beet slices (Stone *et al.*, 1970). Respiration in carrot xylem slices, aerated in 5 mM L-ethionine, was not immediately affected but sensitivity to L-ethionine developed during the ensuing aeration period with maximum effect occurring between 25 and 120 hr (Atkinson and Polya, 1968).

L-Ethionine (25 mM) showed a 78% inhibition of invertase development in sugar beet slices and also inhibited the incorporation of methyl groups from methionine into RNA during the first 6 hr of aeration.

These findings suggest that L-ethionine may act as an effective block of SAM synthesis, hence could be used as a regulator of one-carbon metabolism in such a system.

Although carrots and other storage tissues have been aerated in media containing other amino acids, these studies were generally associated with either uptake or other specific aspects of metabolism. Carrot slices show a capacity to absorb L-alanine and L-aspartate from aerating solutions (Nossier, 1966) and have ability to metabolize methanol, glycine and formate during aeration (Sinha and Cossins, 1964). Radioisotopic feeding experiments carried out by Cossins and Sinha (1965) revealed that feeding of glycine + [^{14}C]formate to carrot slices resulted in a stimulation of ^{14}C incorporation into serine. Feeding of homocysteine + [^{14}C]formate stimulated incorporation of ^{14}C into methionine. It was proposed that formate could serve as a source of C_1 units for the synthesis of serine and methionine via the involvement of folate intermediates. Further investigations to follow up participation of one-carbon metabolism in an aerated storage tissue disk system have not been made.

E. Summary of Major Activities

It is clear from the documented research just presented that storage tissue disks undergo dynamic changes in their metabolism after slicing and aeration. To recapitulate some of the major activities occurring in aerated carrot tissue slices, a graphic presentation has been included (Fig. 1).

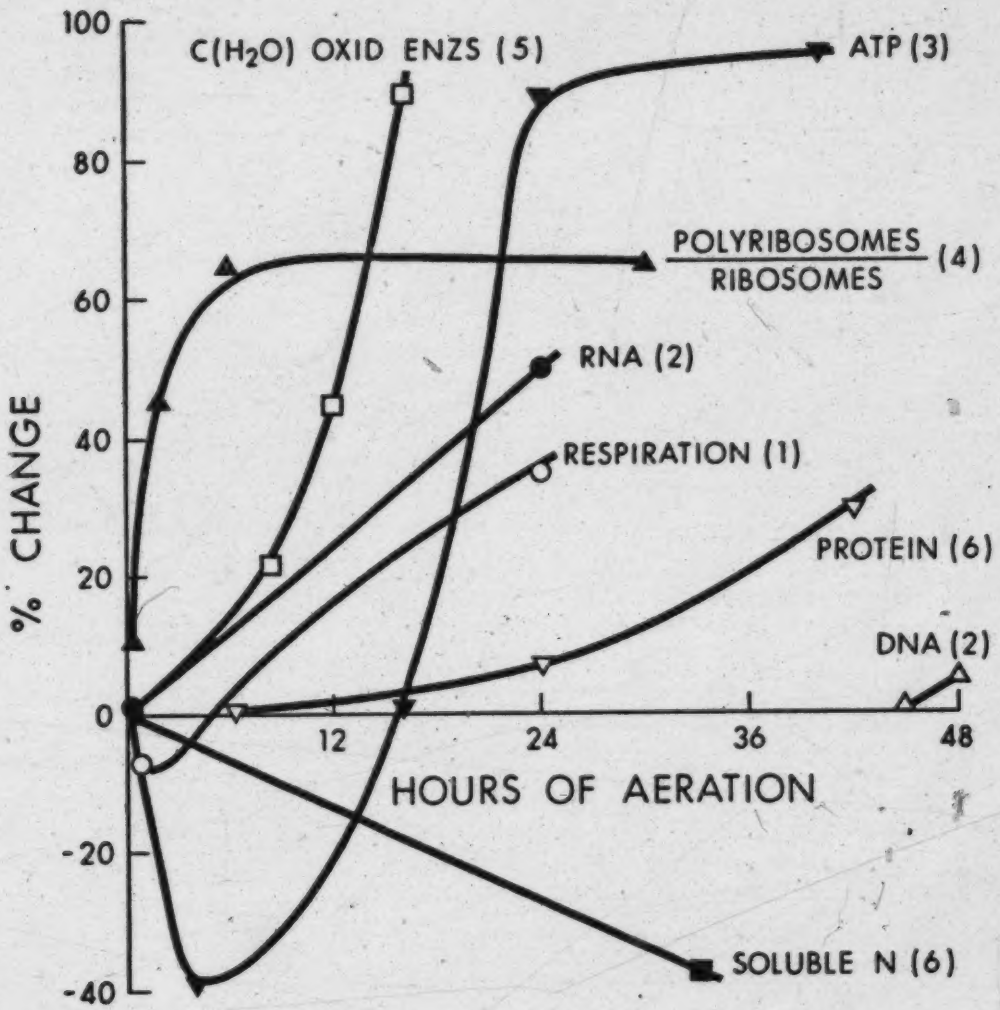


Fig. 1. *Summary of changes in metabolic activity observed in aerated carrot tissue slices*

Source:
1 - Ap Rees and Royston, 1971
2 - Leaver and Edelman, 1965
3 - Adams, 1970*b*
4 - Leaver and Key, 1967
5 - Ricardo and Ap Rees, 1972
6 - MacDonald *et al.*, 1961

II. One-Carbon Metabolism

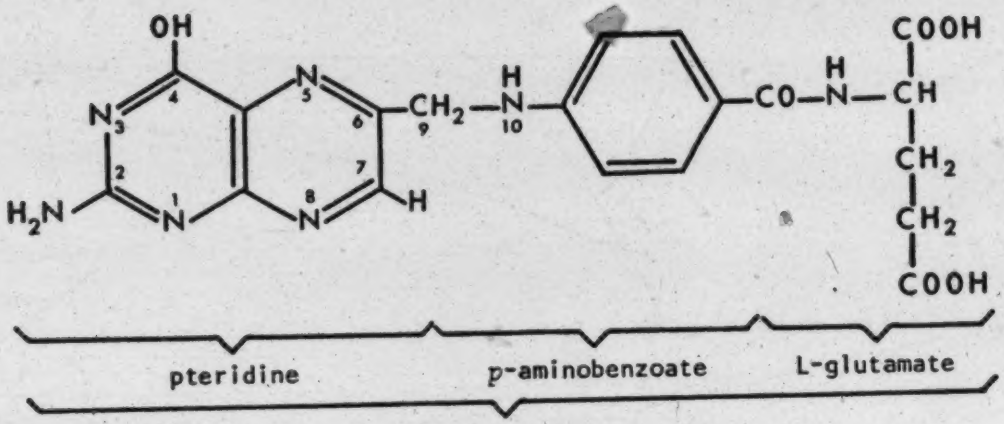
Considerable knowledge has accumulated on the role of folate coenzymes in biosynthetic pathways. Literature dealing with aspects directly related to methyl group biogenesis and its regulation will be emphasized in the review as these have a bearing on the objectives of this study.

A. Historical

Folic acid, or pteroylglutamic acid, has been the object of many detailed investigations in microorganisms, animals and more recently, plants. The nutritional aspects of folic acid as a growth factor in microorganisms has been known for over four decades. Reviews dealing with detailed aspects of folic acid and its biological functions (Stokstad, 1954; Blakley, 1969), assay procedures (Freed, 1966), chemistry (Stokstad, 1954; Whiteley, 1971), occurrence (Toepfer *et al.*, 1951; Blakley, 1969; Baugh and Krumdieck, 1971), metabolism (Rabinowitz and Himes, 1960; Stokstad and Koch, 1967; Blakley, 1969; Ribbons *et al.*, 1970; Jackson and Harrap, 1973) and regulation (Blakley, 1969; Silber and Mansouri, 1971) have been published and may be referred to for further information.

B. Structural Formulae

Folic acid contains a pteridine moiety linked to *p*-aminobenzoate and L-glutamate as represented in the following structural formula:



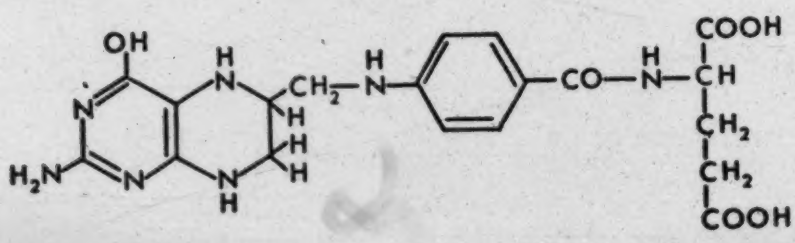
pteroylglutamic acid

folic acid

PteGlu

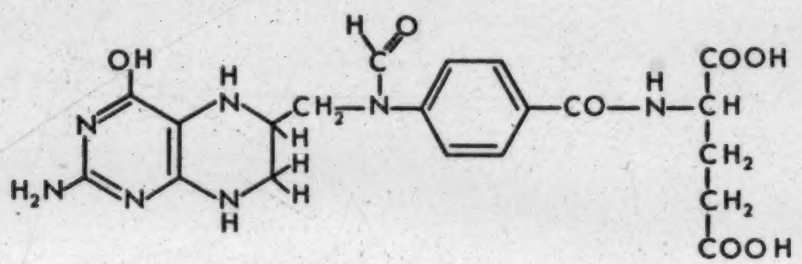
In plant and animal tissues folic acid usually contains in addition more than one glutamyl residue. Such forms are known as conjugates and will be considered later.

An important chemical feature of folic acid is the reducibility of the pteridine ring. Chemical or enzymatic reduction of the pyrazine portion of the ring gives rise to dihydro or tetrahydro derivatives. Tetrahydrofolic acid is one such reduced derivative and in this form is metabolically important as a coenzyme in one-carbon metabolism.

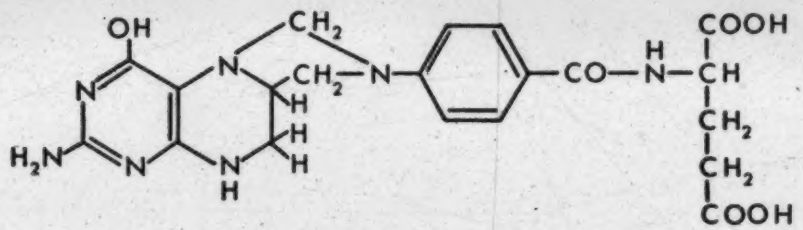


Tetrahydrofolic acid (THFA)

Substitutions, principally at the N^5 and N^{10} positions of the tetrahydrofolic acid molecule, give other metabolically active derivatives such as:

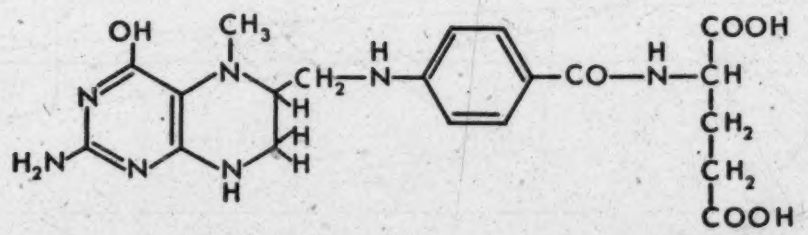


N^{10}-formyl tetrahydrofolic acid,



N^5,N^{10}-methylene tetrahydrofolic acid,

and



N^5-methyl tetrahydrofolic acid.

C. Identification and Estimation of Folic Acid and its Derivatives

Folic acid may be quantitatively assayed by chemical methods but these procedures generally lack the sensitivity necessary to measure the

minute quantities in biological materials. Highly sensitive and specific microbiological techniques have been perfected for this purpose and are now generally employed to distinguish different folate derivatives and to measure their concentrations.

Three organisms are routinely used for assay purposes: *Lactobacillus casei*, *Streptococcus faecalis* and *Pediococcus cerevisiae*. These organisms respond differently to metabolic forms of folic acid and are used to distinguish between the derivatives present. Details of microorganism response may be found in Blakley (1969) or Stokstad and Koch (1967). *L. casei* responds to simpler conjugated forms having less than three glutamic acid residues. *S. faecalis* is used to establish formylated mono and diglutamates while formylated derivatives, especially the N^5 position with one glutamic acid residue, can be assayed with *P. cerevisiae*.

Oxidation of the derivatives occurs readily during preparation and assay unless precautions are taken to maintain reducing conditions as in the 'aseptic plus ascorbate' procedure (Bakerman, 1961; Freed, 1966).

In measurement of total folates in biological tissue, it is common to equate the amount of lactic acid produced by the microorganism to standard amounts of folic acid added to the defined culture medium. Since natural products vary in derivative composition, the phenomenon of 'positive drift' has been encountered with *L. casei* when used to determine total 'free' folates (Tamura *et al.*, 1972). With increasing amounts of assay material, higher values for 'free' folates are commonly obtained. These variations in response are believed to be due to a variable response of *L. casei* to polyglutamate derivatives and Tamura and his co-workers

suggested that an evaluation of 'total free folates' must be considered in light of the presence of polyglutamates or after treatment by carboxypeptidase, an enzyme capable of hydrolyzing the γ-glutamyl peptide bonds of these polyglutamates.

To facilitate estimation of individual folate derivatives, separation by DEAE-cellulose column chromatography is generally employed (Noronha and Silverman, 1962; Bird *et al.*, 1965). The derivatives are sequentially eluted, fractions collected and aliquots of these assayed microbiologically. These techniques have been useful in separating polyglutamates having one to three glutamate residues. For higher polyglutamate derivatives, the recent employment of QAE-Sephadex (Parker *et al.*, 1971) and Sephadex gel chromatography (Shin *et al.*, 1972) has met with considerable success.

D. Polyglutamyl Derivatives and Carboxypeptidase Treatment

As mentioned earlier, folate derivatives exist in plant and animal tissues primarily in the form of polyglutamates. Since assay microorganisms grow on derivatives having one or two glutamic acid residues, the polyglutamates must be hydrolyzed by folic acid 'conjugases' (γ-glutamyl carboxypeptidases) in order to yield forms suitable for microbiological assay. Two types of conjugases are routinely used for this purpose. Most commonly used is a conjugase from chicken pancreas which hydrolyzes polyglutamates to diglutamates (Freed, 1966; Stokstad and Koch, 1967) but other enzymes from hog kidney (Bird *et al.*, 1946) and germinating pea cotyledons (Roos and Cossins, 1971), having ability to hydrolyze polyglutamates to monoglutamates, have been used.

E. Folic Acid and its Derivatives in Biological Tissues

The occurrence of folate derivatives in foods, plants, animals and microorganisms has now been well established. Early studies established the presence of folic acid in foodstuffs (Toepfer *et al.*, 1951; Freed, 1966). Although much exploratory work can be cited regarding early estimation of derivatives in assorted tissue, the comprehensive review of Blakley (1969) presently serves as the best reference. Recent research has established the distribution of derivatives in rat liver (Shin *et al.*, 1972; Thenen *et al.*, 1973), sheep liver (Osborne-White and Smith, 1973), human erythrocytes (Wagner and Levitch, 1973), *Euglena* (Lor and Cossins, 1973) and various higher plant tissues (Iwai and Nakagawa, 1958; Roos *et al.*, 1968; Sengupta and Cossins, 1971; Clandinin and Cossins, 1972; Spronk and Cossins, 1972; Blondeau, 1973; Chan *et al.*, 1973).

With reference to the forms of folate derivatives found in tissues, most folates in animal tissues are polyglutamate forms and have formyl substituents (Noronha and Silverman, 1962; Shin *et al.*, 1972) with pentaglutamates being the most common conjugated form. In plants, however, the major derivatives appear to be methylated (Roos *et al.*, 1968; Chan *et al.*, 1973) with the degree of conjugation varying between tissues; for example, about 50% of pea cotyledon folate is present as the N^5-methylmonoglutamate (Roos and Cossins, 1971) while in cabbage (*Brassica oleracia* var. *capitata*) over 90% of the folate pool is in the form of methyl derivatives having five or more glutamyl residues (Chan *et al.*, 1973).

In tissues more directly related to those of the present investigation, Freed (1966) has reported that fresh carrots contain between

46-76 ng/g fresh wt. of folic acid equivalent as determined by *L. casei*. This level has been termed 'total' folates while 13-49 ng/g fresh wt. has been reported as the 'free' folic acid equivalent in carrot as measured by *S. faecalis*. However, before initiation of the present work, no reports have been made on the nature, regulation or rates of synthesis of individual derivatives in this tissue (Fedec and Cossins, 1972).

F. Metabolic Interconversion of THFA Derivatives

The reaction catalyzing the synthesis of 10-formylTHFA from formate in the presence of ATP and THFA (Fig. 2, Rx. 1) is catalyzed by the enzyme 10-formylTHFA synthetase (EC 6.3.4.3). Activity is commonly enhanced by Mg^{++} and has been detected in several plants (e.g., Hiatt, 1965; Iwai *et al.*, 1967; Clandinin and Cossins, 1972), yeasts (e.g., Lor and Cossins, 1972), *Euglena* (Lor and Cossins, 1973), mammalian tissues (e.g., Sauberlich *et al.*, 1972), liver (e.g., Greenberg *et al.*, 1955), and microorganisms (e.g., Whiteley *et al.*, 1959; Blakley, 1969). Enzyme assay methods have been described by Rabinowitz and Pricer (1963) and Hiatt (1965). In the purine fermentative bacterium *Clostridium*, the reverse reaction has been demonstrated (Curthoys and Rabinowitz, 1972) and the conclusion made that this reaction may serve as a major source of ATP.

The interconversion of 10-formylTHFA and 5,10-methenylTHFA is facilitated by the enzyme methenylTHFA cyclohydrolase (EC 3.5.4.9; Fig. 2, Rx. 2). Most studies have been concerned with beef liver (Greenberg, 1971) and the reaction observed is usually in the direction of 10-formylTHFA after the addition of water to 5,10-methenylTHFA.

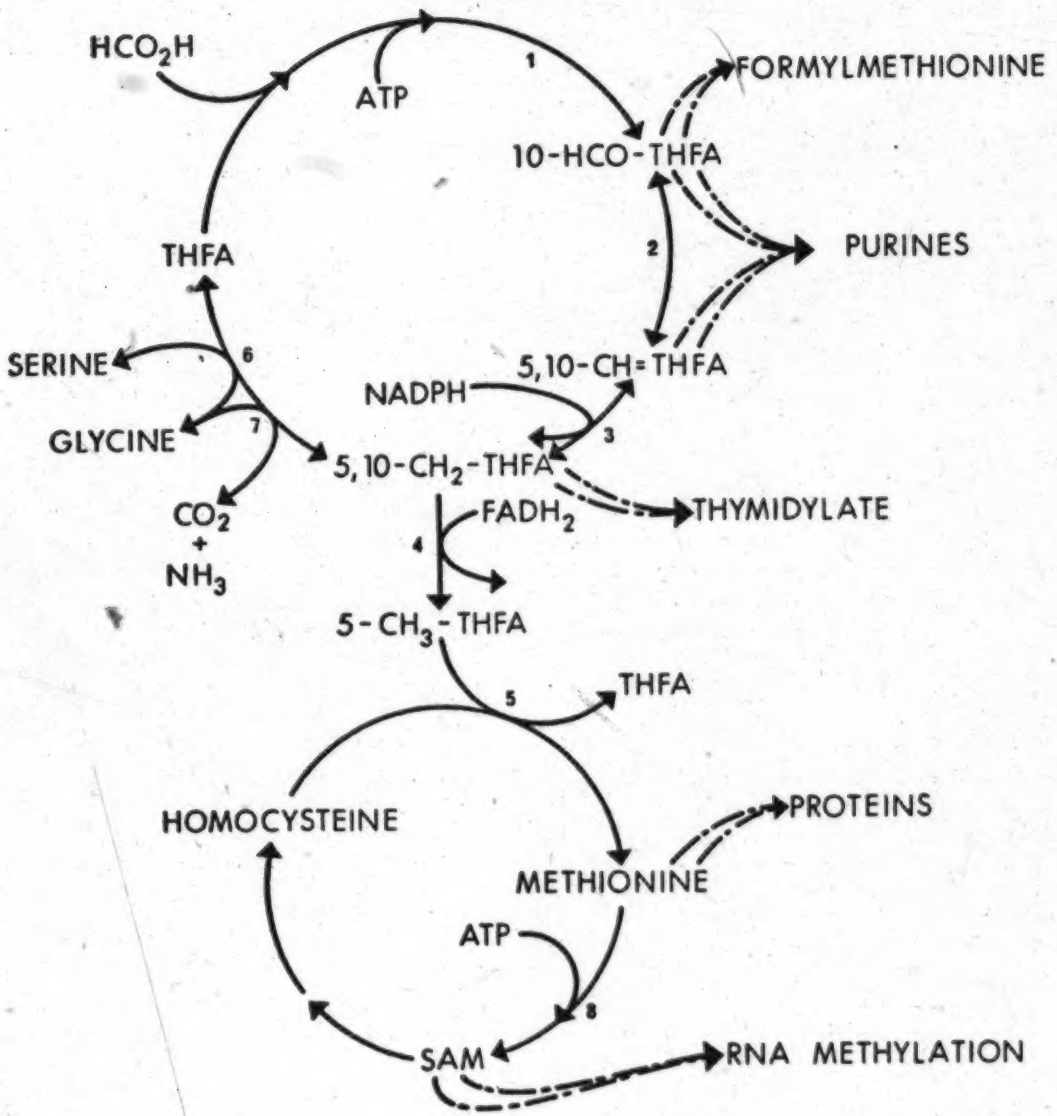


Fig. 2. *Interconversion of THFA derivatives in relation to the generation and utilization of C_1 units*

Synthesis of 5,10-methenylTHFA (Fig. 2, Rx. 3) is facilitated by the enzyme 5,10-methyleneTHFA dehydrogenase (EC 1.5.1.5) from 5,10-methyleneTHFA in the presence of NADP. This enzyme has been studied in mammalian tissues (Osborn and Huennekens, 1957), in plants (Wong and Cossins, 1966) and a variety of microorganisms (Blakley, 1969).

The methylene group of 5,10-methyleneTHFA may be derived from a number of metabolites including formaldehyde, serine, glycine and formate. Reduction of 5,10-methyleneTHFA to 5-methylTHFA (Fig. 2, Rx. 4) by the enzyme 5,10-methyleneTHFA reductase (EC 1.1.1.68) lies at an important branch point of folate metabolism (Kutzbach and Stokstad, 1971). The reaction is dependent upon a source of reduced pyridine nucleotide and is commonly assayed by the reverse reaction in the presence of menadione as an electron acceptor (Donaldson and Keresztesy, 1962). This enzyme occurs in animals (Kutzbach and Stokstad, 1967), yeast (Lor and Cossins, 1972) and higher plants (Cossins *et al.*, 1972).

Synthesis of methionine from 5-methylTHFA and homocysteine is a well established reaction (Fig. 2, Rx. 5) catalyzed by 5-methylTHFA: homocysteine transmethylase (EC 2.1.99). Human tissues (Mudd *et al.*, 1970), microorganisms (e.g., Cauthen *et al.*, 1966; Salem and Foster, 1972), yeast (Lor and Cossins, 1972; Antoniewski and de Robichon-Szulmajster, 1973) and higher plants (Burton and Sakami, 1969; Dodd and Cossins, 1970; Cossins *et al.*, 1972) have been subjected to extensive studies for this enzyme, particularly its regulation as well as its requirement for mono or tri-glutamyl methylated derivatives. Radioisotopic assay of the plant enzyme has made its study relatively easy (Dodd and Cossins, 1970). In micro-organisms and animals a requirement for vitamin B_{12} has been shown (Cauthen *et al.*, 1966; Mudd *et al.*, 1970).

Methionine may also be synthesized by an alternate route from S-adenosyl methionine and homocysteine (Fig. 2, Rx. 8) involving the enzyme SAM:L-homocysteine methyltransferase (EC 2.1.1.e). This reaction which has no requirement for a THFA derivative has been demonstrated in microorganisms (Mardon and Balish, 1971), yeast (Shapiro *et al.*, 1964) and in higher plants (Dodd and Cossins, 1970).

Methionine, produced by either of the above pathways, may be utilized for the production of S-adenosylmethionine (Fig. 2, Rx. 8), the principal donor of methyl groups required in a wide variety of transmethylation reactions. In the presence of ATP, the enzyme methionine adenosyl transferase (EC 2.5.1.6) catalyzes this activation of L-methionine (Ferro and Spence, 1973).

In the transfer of the β-carbon of serine to methionine, the first reaction (Fig. 2, Rx. 6) is carried out by the enzyme serine hydroxymethyltransferase (EC 2.1.2.1); yielding glycine and 5,10-methylene THFA. This enzyme has been extensively studied by various groups (e.g., Wilkinson and Davies, 1960; Botsford and Parks, 1969; Cossins *et al.*, 1972; Schirch *et al.*, 1973). Stability and optimum assay conditions have been described by Nakamura *et al.* (1973) and buffer interactions have been observed by Schirch and Diller (1971). The reaction is freely reversible and consequently may play an important role in the biosynthesis of serine from glycine. For example, recent research has focussed attention on serine synthesized from two molecules of glycine by the combined reaction of glycine decarboxylase (Fig. 2, Rx. 7) and serine hydroxymethyltransferase (Kisaki *et al.*, 1971). More exacting conditions of the former so-called 'glycine cleavage' reaction have been noted in plants by Tolbert *et al.*

(1968), Kisaki *et al.* (1971) and Clandinin and Cossins (1972). Serine hydroxymethyltransferase is commonly a labile enzyme but can be stabilized to some degree by addition of thiol reagents (Schirch *et al.*, 1973), by glycine and serine (Akhtar and El-Obeid, 1972) or by 30% (v/v) glycerol (Nakamura *et al.*, 1973).

G. Regulation of One-Carbon Metabolism

Although the mechanisms responsible for the regulation of one-carbon metabolism have not been fully elucidated, considerable knowledge has been accumulated, particularly in microorganisms (Blakley, 1969). Since this thesis is primarily concerned with methyl group biogenesis, control mechanisms existent in microorganisms, animals and plants related to this aspect can be summarized and more readily visualized in tabular form (Table 1).

From the examples in Table 1 it may be observed that these enzymes are controlled in a wide variety of ways. In general, control is achieved by a product of one-carbon metabolism, e.g., methionine. It must be emphasized, however, that the type or method of control not only varies between organisms but within species as well.

Although most systems studied to date have not been plants, the above examples clearly indicate that methionine must be regarded as a key agent in the control of enzymes which mediate the biosynthesis of methyl groups within the folate pool.

Table 1. *Regulation of enzymes directly related to one-carbon metabolism*

Enzyme	Tissue or organism	Type of control	Agent	Reference
N^{10}-formyl-THFA synthetase	*Micrococcus aerogenes*	Induction	Formate	Whiteley *et al.*, 1959
	Clostridium	Inhibition	N^{10}-HCO-THFA, ADP	Blakley, 1969
	Yeast	Inhibition	Methionine	Lor and Cossins, 1972
	Streptococcus faecalis	Repression	Adenine, guanine	Blakley, 1969
N^5,N^{10}-methylene-THFA dehydrogenase	*Escherichia coli* K_{12}	Inhibition	GTP, ATP	Blakley, 1969
	Salmonella typhimurium	Inhibition	ATP, GTP, GDP	Blakley, 1969
	Streptococcus faecium	Repression	Serine	Albrecht *et al.*, 1968
N^5,N^{10}-methylene-THFA reductase	Rat liver	Inhibition	SAM	Kutzbach and Stockstad, 1971
	Yeast	Inhibition	Methionine	Combepine *et al.*, 1971
	E. coli K_{12}	Repression	Methionine	Silber and Mansouri, 1971
Homocysteine: N^5-methyl-THFA transmethylase	Pea cotyledons	Inhibition	Methionine	Dodd and Cossins, 1970
	Pea root tips	Inhibition	Methionine	Cossins *et al.*, 1972
	Chicken liver	Repression	Methionine	Dickerman *et al.*, 1964
	E. coli	Repression	Methionine	Dawes and Foster, 1971
	Yeast	Repression	Methionine	Lor and Cossins, 1972
Serine hydroxymethyltransferase	Yeast	Induction	Glycine	Botsford and Parks, 1969
	Yeast	Inhibition	Methionine, serine	Botsford and Parks, 1969
	Rabbit liver	Competitive inhibition	Anions	Schirch and Diller, 1971
	Rabbit liver	Inhibition	Thymidine	Nakamura *et al.*, 1973
	Rat liver	Inhibition	N^5-CH_3-THFA	Schirch and Ropp, 1967
	E. coli	Repression	Methionine	Nakamura *et al.*, 1973
SAM synthetase	Yeast	Repression	SAM	Ferro and Spence, 1973

III. Interrelationships between the Aging of Storage Tissue Disks and One-Carbon Metabolism

A. The Basic Prerequisite for C_1 Metabolism in this System

Fig. 2 emphasizes those areas of metabolism in aerated tissue slices which would logically have requirements for C_1 units derived from precursors such as formate, glycine or serine.

Since a basic characteristic of aerated tissue slices is their enhanced rate of net protein synthesis (MacDonald *et al.*, 1961), it follows that protein amino acids including methionine must be available. In the case of methionine, a net synthesis would conceivably involve not only generation of homocysteine but production of methyl groups from the folate pool as well.

Before such new proteins can be produced, there must also be a synthesis of RNA and DNA. It is well documented that such syntheses occur in aerated tissue slices (Ellis and MacDonald, 1967; Bryant and Ap Rees, 1971). Considering the magnitude of these latter syntheses, it is clear that RNA and DNA will be formed from their constituent bases which in turn will be derived by folate-mediated reactions.

King and Chapman (1973) working with artichoke tissue slices have shown that during aeration an increased methylation of tRNA occurs. In barley aleurone, such methylation of RNA is increased by application of GA_3 (Chandra and Duynstee, 1971). This growth hormone also increased the activity of tRNA methylase in this system (Chandra, 1970). Clearly, by analogy to bacterial and animal systems, it follows that the required

methyl groups would be provided by SAM which would in turn derive these from methyl folates via methionine.

In conclusion, it must also be pointed out that before synthesis of proteins would occur in aerated disks, initiation of the polypeptide chain must occur. In this regard, formylmethionine (Fig. 2) plays a primary role with 10-formylTHFA being the major formyl donor (Dickerman, 1971).

B. The Present Investigation

It needs no further emphasis that aeration of storage tissue disks is accompanied by dynamic changes in several physiological and biochemical processes (Fig. 1) and that C_1 metabolism must play a central role during this period (Fig. 2). The present investigation, therefore, sought to:

(a) establish the nature of the folate pool in this system,

(b) observe the changes occurring in the pool as affected by treatment and aeration,

(c) establish the activity of the enzymes involved, and

(d) investigate ways by which reactions of importance to methyl group biogenesis might be regulated.

The results of these investigations in relation to the established biochemical changes which are initiated by slicing and aeration of storage tissue disks will be discussed.

MATERIALS AND METHODS

I. Materials

A. Chemicals

Sodium-[^{14}C]formate, [*Me*-^{14}C]-5-CH_3-tetrahydrofolate and L-[3-^{14}C]serine were purchased from Amersham-Searle Corp., Des Plaines, Il., U.S.A. Purity of these labelled compounds was checked by thin-layer chromatography or column chromatography according to methods suggested by the manufacturer. Other chemicals used, of highest quality commercially available, were purchased from Canlab, Edmonton, Alta.; Fisher Scientific Co., Edmonton, Alta.; ICN Pharmaceuticals, Inc., Cleveland, Oh., U.S.A. and Sigma Chemical Co., St. Louis, Mo., U.S.A. Tetrahydrofolic acid was purchased exclusively from Sigma Chemical Co. Scintillation grade 2,5-diphenyloxazole (PPO) and 1,4-*bis*-[2-(4-methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP) were purchased from Amersham-Searle Corp., Des Plaines, Il., U.S.A.

B. Plant Material

In preliminary studies, carrot roots (*Daucus carota* L.) were either purchased from the local market or grown in the home garden. In studies reported here, carrots of the Nantes Coreless variety were grown on a continuous basis, in the Dept. of Botany growth chambers. Seeds were planted in 3:2:1 soil mix in specially constructed 12" x 15" x 12" plywood boxes. Water was added as required. A 3000 ft candle light source (fluorescent and incandescent combined) was used to provide a 14 hr day. Day temperature was maintained at 24°C and night temperature was 20°C.

After a five-month growing period, carrots were harvested, topped, washed, rinsed twice in distilled water, blotted dry, packaged into cellophane bags and stored in the dark at 4°C.

Carrots used for experimental purposes had been stored for a minimum of 1 month but not longer than 3 months.

II. Methods

A. Preparation of Carrot Slices

Carrots from storage were washed in soapy water and rinsed 4 times with sterile distilled water. All equipment used in preparing carrot slices was previously autoclaved or washed in 96% ethanol. Sterile distilled water was routinely used for all rinsing of tissues and in all aeration procedures. All treatment solutions, such as aminopterin, GA_3, L-methionine and L-ethionine, were passed through 0.22 µm Millipore filters (Millipore, Montreal, P.Q.) before use in experiments.

Sections of carrot root, 55 mm in length, were cut to fit a mechanical guillotine specially designed for multiple cutting of tissue slices. Corings of carrot phloem parenchyma were made with a #2 cork borer giving 9 mm diameter cores. Cores were then placed into a beaker of sterile distilled water. After the necessary number of cores for a particular experiment had been prepared, random selection was made for slicing. The guillotine cut over 250 - 1 mm x 9 mm disks from a single batch of 5 cores inserted into the machine. A small sample of each batch was taken to make up the control treatment or the zero time sample, wherever applicable. The disks were rinsed 3 successive times with sterile distilled water before being transferred to aerating flasks.

B. Aeration of Carrot Slices

Aeration or aging (the term commonly utilized in current literature) of the slices was carried out in pre-sterilized Erlenmeyer flasks of either 250 or 500 ml capacity depending upon the quantity of slices required for a particular treatment. The quantity of aging solution was maintained in the proportion of 4 disks/ml of aerating solution.

Aeration was facilitated by evacuating the flasks, thereby drawing sterile, moisturized air into the flask through a fritted glass aerator. This caused a gentle bubbling action in the solution and at the same time created a circulating action. Where disks were aerated for more than 24 hr, the solution was replaced by fresh sterilized solution every 12 hr.

C. Tissue Sampling

Disks were harvested from the aerating flasks with a sieve cup stainless steel dipper. Sterile techniques were utilized throughout. Samples of 50 or 100 disks were taken, blotted twice with paper towelling and weighed.

D. Bacterial Contamination of Carrot Tissue Disks

Periodic checks for the presence of contaminating bacteria in the aerating solutions were made. Serial dilutions of the medium were plated out after 24 and 48 hours of aeration after solution changes had been made every 12 hr or every 24 hr. Agar plates of tryptone and yeast extract were used as the media. After the plates were incubated for 24 hr at 37°C, colony counts were taken as a measure of bacterial contamination.

E. Respiration of Tissue Disks

Carrot disks, prepared in the usual manner, were aerated for a 48 hr period in the following media: distilled water control, 0.1 mM GA_3, 5 mM L-methionine, 10 mM L-ethionine and 0.02 mM aminopterin. Samples for measurements of respiration were taken after 0, 1, 3, 6, 12, 24, 36, 48 hr. Duplicate samples of 5 disks each were then placed in 5 ml of the respective solution and rates of oxygen uptake determined at 25°C using a YSI No. 53 Biological Oxygen Monitor (Yellow Springs Instruments Co., Yellow Springs, Oh., U.S.A.). Solubility of oxygen in the various media used was taken from known absorption coefficients of dissolved oxygen or directly determined by the Winkler method (Natelson, 1957).

F. Analysis of Folate Derivatives

Extraction of tissue to insure maximum isolation of folate derivatives was followed as outlined by Roos and Cossins (1971). Disks were immersed in 10 ml of 2% K-ascorbate (pH 6.0) at 95°C for 10 min. After cooling in an ice bath, transfer was made to a 15 ml Virtis homogenizer flask and the disks were homogenized at 50,000 rpm for 1.5 min using a Virtis (model 45) homogenizer. The homogenate was quantitatively transferred to a 30 ml centrifuge tube and centrifuged for 10 min at 18,000*g*. The supernatant was collected in a test tube and the pellet resuspended in 5 ml 1% K-ascorbate and recentrifuged. The supernatants were combined, made up to 20 ml with 1% K-ascorbate and stored at -20°C until required.

Column chromatography of the folate derivatives was performed using DEAE-cellulose columns (20 cm x 1.8 cm) and a continuous convex

gradient elution using a 0 to 0.5 M K-phosphate buffer (pH 6.0) in the presence of ascorbate (Sotobayashi *et al.*, 1966). Folate derivatives were identified using the basic criteria of differential growth response of microorganisms as described earlier (Roos *et al.*, 1968; Roos and Cossins, 1971). As a general practice, fractions were not collected beyond 120 although periodic checks were made for (polyglutamate) derivatives by extending collection to 200 fractions (total elution volume of 600 ml).

Microbiological assay of the folate derivatives was carried out according to procedures outlined by Bakerman (1961) and Roos and Cossins (1971). *Lactobacillus casei* (ATCC 7469), *Pediococcus cerevisiae* (ATCC 8081) and *Streptococcus faecalis* (ATCC 8043), cultured according to Freed (1966), were routinely used to determine methylated and formylated derivatives. Standard reference curves were prepared using either folic acid (Sigma Chemical Co.) or 5-formylTHFA (American Cyanimid Co., Pearl River, N.Y., U.S.A.). The production of lactic acid by the microorganisms after 72 hr at 37°C was backtitrated and used as a measure of growth (Freed, 1966). Titration values were compared to constructed standard curves and used in estimation of folate content.

For enzymatic hydrolysis of polyglutamates, γ-glutamyl carboxypeptidase was prepared from pea cotyledons by a method similar to that of Roos and Cossins (1971). Ten grams of 3-day old germinating pea cotyledons were ground at 4°C in 8.0 ml of 0.1 M potassium phosphate buffer (pH 6.0) in a mortar by hand. The cellular debris was removed by squeezing the slurry through several layers of cheesecloth and the liquid centrifuged for 20 min at 18,000*g*. The supernatant was dialyzed for 16 hr at 4°C against 2 litres of phosphate buffer. This was then made up to 40 ml,

dispensed into 5 ml volumes and frozen. When used, the enzyme was diluted with about 5 volumes of 0.1 M Na-acetate buffer (pH 5.0) plus 1.0% ascorbate. This was equivalent to 1.5 mg protein/ml as measured by the method of Lowry *et al.* (1951). The quality of the γ-glutamyl carboxypeptidase was routinely checked by testing its ability to hydrolyze the polyglutamates of commercial yeast extract (Roos and Cossins, 1971). Time of incubation was 10 hr at 35°C. Boiled enzyme controls were routinely included.

It is recognized that difficulty in estimating total folate content in an extract has been experienced (Tamura *et al.*, 1972). In the present investigations, to bring about some uniformity of results, values for total folates were calculated from dilutions in a range where aliquot volume was a linear function of folate content.

G. Analysis of Free and Protein Amino Acids

Free amino acids were extracted from carrot slices using the methods of Cossins and Beevers (1963) and Splittstoesser (1969). At sampling time 100 carrot disks were weighed and immediately placed in 10 ml of boiling 80% ethanol for 5 min. These were then quantitatively transferred to a 20 ml Virtis flask and homogenized for 1.5 min at 50,000 rpm. The homogenate was transferred to a glass centrifuge tube and centrifuged for 15 min at 18,000*g*. After decanting the supernatant, the pellet was resuspended in 7 ml of 50% ethanol and recentrifuged. The pellet was washed a third time in 7 ml distilled water. The supernatants were combined and flash evaporated to dryness at 35°C. The residue was redissolved in 5 ml of distilled water and applied to a 1 cm x 6 cm column of Dowex 50W-X8 (H^+, 200-400 mesh; BioRad Labs., Richmond, Ca., U.S.A.). After passing 50 ml of distilled water through the column to

elute sugars and organic acids, 40 ml of 4 M HCl were used to elute the amino acids. After flash evaporating this eluate at 40°C, the residue was redissolved in 5 ml of 0.067 M sodium citrate buffer (pH 2.2) and frozen at -30°C until analysis could be conducted.

Amino acids were chromatographed using a Beckman Automatic Amino Acid Analyzer Model 121 (Beckman-Spinco Instruments, Inc., Palo Alto, Ca., U.S.A.) by the method of Moore and Stein (1963). An 8 cm bed of Beckman-Spinco P.A. 35 resin was used for basic amino acid separation and a 54 cm bed of Beckman-Spinco P.A. 28 resin was used for neutral and acidic acid separation. Buffer elution sequence was identical to that of Clandinin and Cossins (1972).

In the preparation of protein amino acids, 50 mg samples of the dried pellet remaining after centrifugation in the preparation of plant extracts were weighed out into 16 mm x 135 mm heavy-walled Pyrex test tubes. After suspension of the material in 5 ml of 6 M HCl, the tubes were placed in a dry ice-acetone bath. After freezing, the sample container was evacuated to 50 microns and sealed. Hydrolysis was conducted in an oil bath heated to 145 ± 2°C for 4 hr (Roach and Gehrke, 1970). After cooling, the hydrolyzate was filtered through a 15 ml 10-15 μm sintered glass funnel and the HCl removed *in vacuo*. The residue was taken up in 10 ml distilled water, applied to a 1 cm x 6 cm Dowex 50W-X8 (H^+, 200-400 mesh) resin column and processed as outlined for amino acid extract preparation.

Similarly, selected protein pellets, from the [^{14}C]formate feeding experiment, were hydrolyzed and analyzed for labelled protein amino acids using the automatic amino acid analyzer.

H. Enzyme Studies

1. Preparation of cell-free extracts

Aerated carrot tissue slices were homogenized in 0.05 M potassium phosphate buffer (pH 6.9) containing 2 mM 2-mercaptoethanol in a Virtis homogenizer at 4°C. Cell debris was removed by centrifugation (18,000*g* for 20 min) and the supernatant retained for subsequent enzyme assays. In a number of experiments, these enzyme extracts were desalted by passage through 1.5 cm x 7 cm columns of Sephadex G-15 at 4°C (Pharmacia Fine Chemicals, Montreal, P.Q.).

2. Enzyme assays

N^5,N^{10}-Methylenetetrahydrofolate reductase (EC 1.1.1.68) activity was assayed on the basis of menadione-dependent oxidation of [*Me*-^{14}C]-5-CH_3-THFA to THFA and [^{14}C]formaldehyde (Donaldson and Keresztesy, 1962). The complete reaction system contained: 10 µmol potassium phosphate buffer (pH 6.4), 5 nmol FAD, 5 nmol menadione, 5 µmol formaldehyde, 1.6 nmol [*Me*-^{14}C]-5-CH_3-THFA (1 µCi/16 nmol) and enzyme to a total volume of 0.32 ml. After incubation at 30°C for 30 min, the reaction was terminated by rapid cooling in an ice bath. The cooled reaction mixture was placed on a 0.5 cm x 2.5 cm column of Dowex AG1-X10 resin (Cl^-, 200-400 mesh). The [^{14}C]formaldehyde was eluted with three washings each of 0.5 ml distilled water and collected directly in a scintillation vial for counting.

N^5-Methyltetrahydrofolate:homocysteine transmethylase (EC 2.1.99) activity was assayed by the method of Dodd and Cossins (1970). The standard reaction mixture contained: 1 µmol of L-homocysteine freshly prepared from the thiolactone form, 1.6 nmol [*Me*-^{14}C]-5-CH_3-THFA (1 µCi/16 nmol), 50 µmol

potassium phosphate buffer (pH 6.3) and cell-free extract to a total of 0.5 ml. After incubation at 30°C for 30 min, the reaction was terminated by rapid cooling in an ice bath. The cooled reaction mixture was placed on a 0.5 cm x 2.5 cm column of Dowex AG1-X10 resin (Cl^-, 200-400 mesh). The [*Me*-^{14}C]methionine synthesized was eluted with six washings each of 0.2 ml distilled water and collected directly in a scintillation vial for counting. The amount of methionine produced was determined from the specific radioactivity of the substrate used.

Serine hydroxymethyltransferase (EC 2.1.2.1) activity was assayed by the method of Taylor and Weissbach (1965), with minor modification. This method is based on the principle that since the radioactive C_1 unit of 5,10-methyleneTHFA equilibrates readily with carrier formaldehyde, it can be trapped as the 5,5-dimethyl-1,3-cyclohexadione adduct and quantitatively estimated. The complete reaction system contained: 1.77 nmol L-[3-^{14}C]serine (1 µCi/17 nmol), 0.1 µmol pyridoxal-5-phosphate, 30 µmol potassium phosphate buffer (pH 8.0), 1.6 µmol THFA, 2 µmol 2-mercaptoethanol and enzyme in a total of 0.4 ml. All components, except serine were pre-incubated for 5 min at 30°C. The reaction was initiated by the addition of substrate and terminated after 15 min at 30°C by the addition of 0.3 ml 1 M sodium acetate (pH 4.5), 0.2 ml 0.1 M formaldehyde and 0.3 ml 0.4 M 5,5-dimethyl-1,3-cyclohexadione in 50% (v/v) ethanol. The HCHO-dimedon derivative was formed by heating for 5 min at 100°C. After cooling the adduct was extracted in 3.0 ml toluene of which a 0.5 ml aliquot was counted for radioactivity.

N^{10}-Formyltetrahydrofolate synthetase (EC 6.3.4.3) activity was assayed by the method of Hiatt (1965). During preparation of cell-free

extracts the extraction buffer included 0.1 mM GSH as an additional constituent. The complete reaction system contained: 100 µmol triethanolamine buffer (pH 8.0), 150 µmol Tris-formate (pH 8.0), 2.5 µmol $MgCl_2$, 200 µmol KCl, 4 µmol THFA, 2 µmol ATP, 10 µmol 2-mercaptoethanol and enzyme to a total volume of 0.8 ml. After incubation for 15 min at 30°C, the reaction was terminated by addition of 1 ml 1 M HCl. Absorbance at 355 nm was measured after 10 min and the amount of 5,10-methenylTHFA formed was calculated from the molar extinction coefficient of 22 x 10^6 cm^2 (Bertino *et al.*, 1962).

3. Expression of enzyme activity

Enzyme activities reported in this study are expressed as the amount of product produced under the reaction conditions/hr/mg of protein present in the cell-free extract.

4. Protein assay

The protein content of cell-free plant extracts was estimated spectrophotometrically using the method of Lowry *et al.* (1951). Crystalline egg albumin (ICN Pharmaceuticals, Inc., Cleveland, Oh., U.S.A.) was used as the reference standard and determinations were made in duplicate using a Beckman DB-G spectrophotometer (Beckman Instruments).

I. [^{14}C]Formate Feeding

Batches of carrot slices were prepared in the usual manner and aerated for periods of up to 48 hr in treatments including distilled water, 0.1 mM GA_3 and 5 mM L-methionine. Twenty-five disks were harvested from each treatment at 12, 24, 36 and 48 hr intervals of aeration. A zero time

control treatment was also included. The disks were transferred to Warburg flasks containing 1 µmol [^{14}C]formate (2.5 µCi/µmol; pH 5.9) in 4.0 ml distilled water. The centre well contained a 2.5 cm x 3 cm filter paper saturated with 20% KOH.

The disks were incubated with [^{14}C]formate for 1 hr at 30°C in a shaking water-bath. A non-tissue blank flask containing [^{14}C]formate and KOH-impregnated filter paper was also included to check release of ^{14}C from the incubating solution. $^{14}CO_2$ values were then corrected by this figure which usually ranged from 7000-10000 c.p.m.

After incubation, disks were harvested, washed with distilled water to remove excess labelled formate and plant extracts prepared for fractionation into organic acids, sugars and amino acids.

Normally, 25 carrot slices from the feeding experiment were transferred to 10 ml of boiling 80% ethanol for 5 min. These were then transferred to a Ten Broeck tissue homogenizer and ground manually for 10 minutes by which time a fine homogeneous preparation was obtained. The homogenate was quantitatively transferred to a glass centrifuge tube and centrifuged at 18,000*g* for 15 min. The supernatant was decanted into an evaporating flask and the pellet resuspended in 10 ml 50% ethanol followed by recentrifugation. A third pellet wash was carried out with 10 ml distilled water. The combined supernatants were flash evaporated to dryness at 35°C. The residue after flash evaporation was redissolved in 10 ml of sterile distilled water. Lipids were extracted with 3 ml toluene and 2 ml petroleum ether and an aliquot counted by liquid scintillation spectrometry.

For purposes of fractionation, all of the plant extract was applied to a 2 cm x 5 cm column of Dowex 50W-X8 (H^+, 200-400 mesh) resin and the sugars and organic acids eluted with 50 ml of distilled water. The amino acids were eluted with 40 ml of 4 M HCl and designated the 4 M amino acid fraction. The column was further washed with 40 ml of 6 M HCl and this designated the 6 M amino acid fraction. The amino acid fractions were flash evaporated to dryness under vacuum at 40°C. Ten ml of distilled water were added and this was flash evaporated off. Three such additional rinses were carried out to facilitate removal of the residual HCl. The 4 M fraction residue was redissolved in 5 ml of sterile distilled water and the 6 M fraction into 3 ml of sterile distilled water. Aliquots of each were counted for radioactivity and the sample further processed for amino acid analysis and/or paper chromatography.

The organic acids and sugars which had been collected initially were separated using a 2 cm x 5 cm column of Dowex 1W-X10 (Cl^-, 200-400 mesh) resin converted to the formate form (Splittstoesser, 1969). After applying the organic acid-sugar mixture, the column was eluted with 40 ml of distilled water to bring off the sugars. Next, the column was eluted with 40 ml of 4 M formic acid to elute the organic acids. The sugar solution was adjusted to pH 1.0 with 6 M HCl and then flash evaporated at room temperature to 25% of the original volume in order to volatilize any residual [^{14}C]formate that may have carried through from the feeding pulse. The solution was adjusted back to pH 6.5 and an aliquot counted for radioactivity. The organic acids were evaporated, under vacuum, to dryness at 35°C, redissolved in 5 ml of distilled water and an aliquot counted for radioactivity.

Amino acids labelled after ^{14}C feeding were separated primarily by descending paper chromatography. Samples were applied in duplicate to 60 cm x 60 cm Whatman No. 1 paper. The papers were placed into glass chromatography tanks and developed (40 cm, one dimension descending manner) in phenol-water-ammonia (160:40:1, by vol.). After removal of the solvent in a vented chromatography oven at room temperature, one of the duplicates was sprayed with ninhydrin solution (Sigma Chemical Co.) and heated to 100°C for 5 min. The amino acid areas were then compared to known standards. The comparable areas on the other developed chromatograms were cut out, placed into liquid scintillation vials and their radioactivity determined. Counts were corrected for quenching by the use of external standard methods.

For more complete data of amino acid labelling, samples were examined for ^{14}C after analysis in the Beckman-Spinco 121 analyzer. Fractions of 1.9 ml were collected from the analyzer and the elution pattern verified by reference to reaction with ninhydrin reagent. This was further established by addition of [^{3}H]methionine and [^{14}C]serine to the amino acid standard. Aliquots of each fraction were then counted for radioactivity in liquid scintillation vials previously filled with fluor and precounted for background. The remainder of the eluate fraction was reacted with ninhydrin to visualize positions of amino acid peaks.

J. Assay of Radioactivity

Radioactive samples were counted in a liquid scintillation counter (Model Unilux II, Nuclear-Chicago Corp., Des Plaines, Il, U.S.A.). Aliquots of up to 1.5 ml aqueous sample were counted in 15 ml of fluor

containing 6.5 g 2,5-diphenyloxazole (PPO) and 0.65 g 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP)/litre of dioxane:anisole:dimethoxyethane (6:1:1, by vol.). The counting efficiency of the above system was 85% as determined by addition of internal standards. Quench correction was made by using external standard quench correction methods. All counts were corrected for background and regarded as significant only if they were at least double this level in duplicate.

Radioactive areas from paper chromatograms were counted by cutting out the spots and placing them into scintillation vials with 15 ml of fluor.

RESULTS

In designing experiments to investigate the objectives set out in this thesis, namely: (a) establishment of the nature of the folate pool and its changes in a system using aerated storage tissue slices, (b) activity of C_1 enzymes, and (c) regulation of methyl group biogenesis, aeration of carrot tissue slices in distilled water was considered as the standard control. In order to alter the flow of one-carbon units and hence facilitate achieving the above objectives, carefully chosen compounds were added to the aerating slices.

The compounds chosen for the investigations reported herein were: (a) GA_3, a growth-regulating substance having ability to enhance methylation, nucleic acid synthesis, respiration in storage tissue disks and synthesis of enzymes, (b) L-methionine, a key end product of C_1 metabolism and known for its role in biological methylations, regulation of several C_1 enzymes and as an inhibitor of respiration in some storage tissue disks, (c) L-ethionine, a methionine analogue responsible for inhibition of both storage tissue disk respiration and methyl group incorporation from methionine into RNA, and (d) aminopterin, an antimetabolite and folic acid antagonist.

The results reported in this Section also consider bacterial contamination of the aerating solution and the source of tissue, the latter from the standpoint of storage effect on parameters readily observed such as respiration and amino acid pools. Of particular interest, in this regard, were amino acids which serve as substrates and end products of one-carbon metabolism. These two areas will be considered first.

These results will be followed by the effects of aeration and treatment on folate levels, enzymes of C_1 metabolism, specific effects of L-methionine *in vitro* and studies of [^{14}C]formate metabolism.

1. Bacterial Contamination of Carrot Tissue Slices during Aeration

The question of bacterial contamination comes foremost into mind when analysis of folic acid and its derivatives is being considered since bacteria contain, in proportion to weight, relatively high quantities of folates. THerefore, one must be certain that values obtained for tissue disks are truly representative of folate derivatives in the tissue and not due to folates from build-up of bacteria in the medium during the aging or aeration period. Since bacteriostats interfere not only with the microbiological assay of folate derivatives but also with the metabolic processes occurring in the tissue slices (Ap Rees and Bryant, 1971) use of these compounds was precluded, but an attempt was made to aerate the tissue slices in such a way that contamination would be minimized. Using routine aseptic measures, colony counts (Table 2) were within an acceptable range after 24 hr of aeration but became very high by 48 hr in some of the treatments; namely, ethionine and aminopterin. After filtering the aerating solutions through micro-porous filters, bacteria counts were reduced considerably. These levels of bacteria were within the limits considered tolerable by Edelman and Hall (1965). The data indicate that bacterial contamination was due mainly to bacteria in the chemical ingredients rather than in the tissue.

Subsequent aeration procedures utilized sterile distilled water or micro-porous filtered solutions coupled with change of the aerating

Table 2. *Estimation of bacterial contamination in the aerating medium*

Aliquots taken, after aging periods shown, were incubated on nutrient agar plates for 24 hr at 37°C. 600 carrot slices (1 mm x 9 mm)/250 ml medium. The solutions were changed every 24 hr. A, sterile distilled water used for the control and the solutions; B, sterile distilled water used for the control but the solutions were passed through 0.22 µm Millipore filters.

Treatment	10^3 Colonies/250 ml medium	
	A	B
After 24 hr aeration:		
Distilled water control	4.3	3.5
0.1 mM GA_3	30.2	14.6
5.0 mM L-methionine	23.8	13.4
10.0 mM L-ethionine	16.3	14.1
0.02 mM aminopterin	59.4	17.3
After 48 hr aeration:		
Distilled water control	95.6	86.3
0.1 mM GA_3	205.0	118.5
5.0 mM L-methionine	423.1	178.9
10.0 mM L-ethionine	2500.0	467.5
0.02 mM aminopterin	1593.8	397.0

solution every 12 hr if the aeration period exceeded 24 hr. Routine checks were made of the aerating solutions to ensure that these standards were maintained. Generally, bacterial counts were well below those shown in Table 2 and were often as low as 500 colonies/250 ml in the distilled water control and 1875 colonies/250 ml in the methionine treatment after 24 hr of aeration.

II. Measurement of Carrot Slice Respiration and Standardization of Tissue Source

After making initial studies of amino acid levels in carrot tissue during post-harvest storage and during aeration of carrot tissue slices, it became abundantly clear that reproducible results could not be obtained from tissue obtained from different sources. It was, therefore, decided that a source of consistent tissue must be secured. The best approach was cultivation of carrots in the growth chamber on a rotating basis, storage of them for a designated period and then use in subsequent studies. Respiratory rates of carrot tissue obtained from different sources are shown in Fig. 3. Slices from freshly harvested carrot had a much higher respiration rate at the time of slicing than slices prepared from tissue stored at 4°C for 2 months. All tissue showed a decline in the respiration rate immediately after slicing although 4 hr of aeration overcame this. Aerated slices from freshly harvested carrot did not exhibit a dramatic rise in respiration rate. Similarly, slices of greenhouse-grown carrot stored for 2 months did not show as dramatic a rise in respiration rate as did material 'purchased locally' (Fig. 3). The latter material behaved similarly to that of Adams and Rowan (1970).

Fig. 3. *Effect of aeration on respiration of slices cut from carrots obtained from different sources*

Disks (1 mm x 9 mm) were cut from carrots and aerated in sterile distilled water. Two replicates of 5 disks each were sampled and the oxygen uptake determined by a YSI oxygen monitor. Fresh wt. of the disks was determined after an 8 min run. The mean values were plotted for slices cut from greenhouse-grown carrots stored for 2 months at 4°C (O), greenhouse-grown carrots freshly harvested (■) and 'locally purchased' carrots, storage conditions unknown (▲).

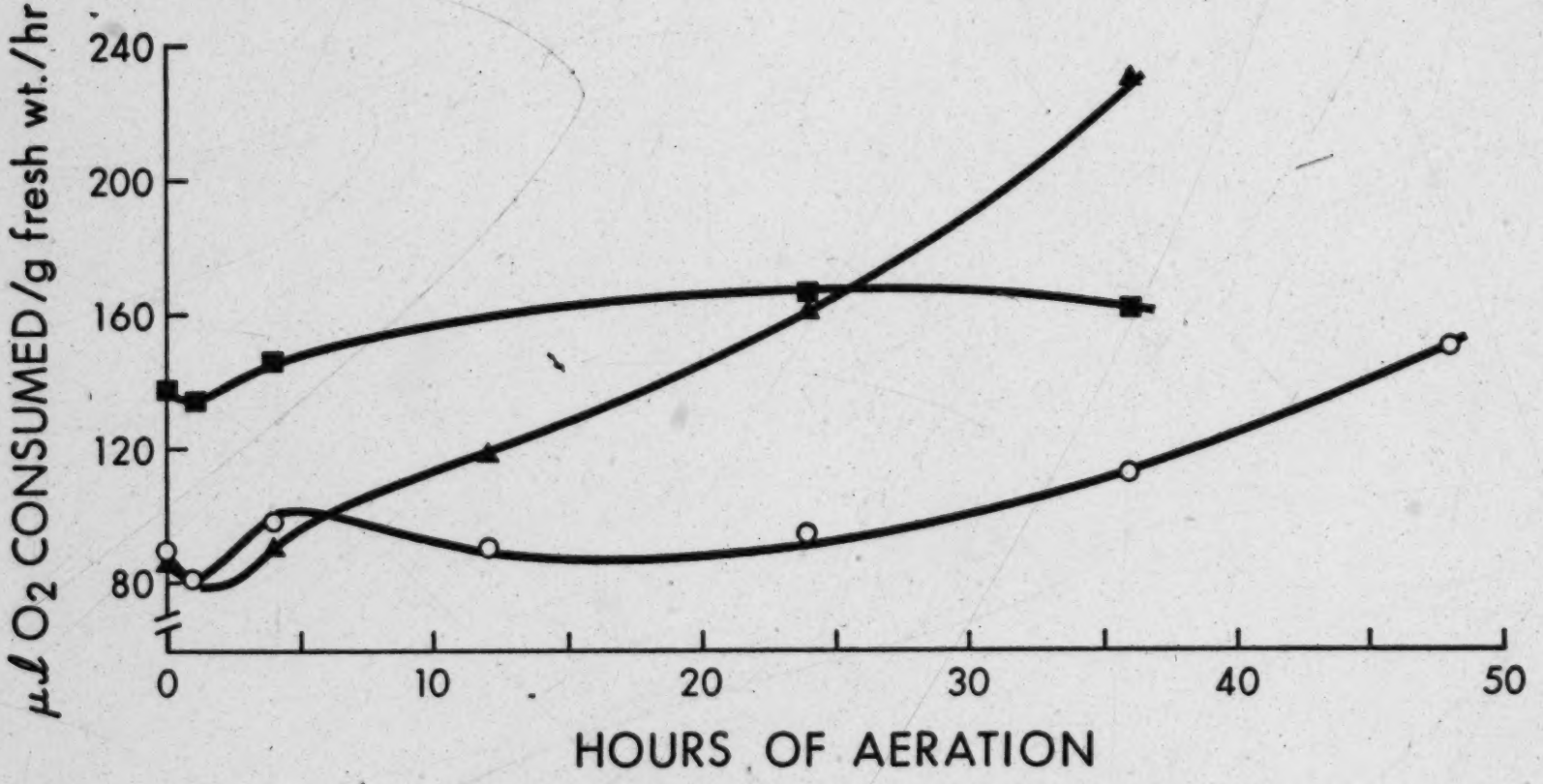

µl O2 CONSUMED/g fresh wt./hr
240
200
160
120
80
0
10
20
30
40
50
HOURS OF AERATION

Fig. 4 (A and B) shows the respiration rate of carrot slices as a function of aeration in distilled water (control) and in various treatments used throughout this study. A different source of greenhouse-grown tissue was used in the two experiments and it is noted that the initial respiration rate of disks in the second experiment (Fig. 4B) was higher than for those in Fig. 4A. However, following a decline in respiration after slicing, the induced respiration rates of both controls were similar.

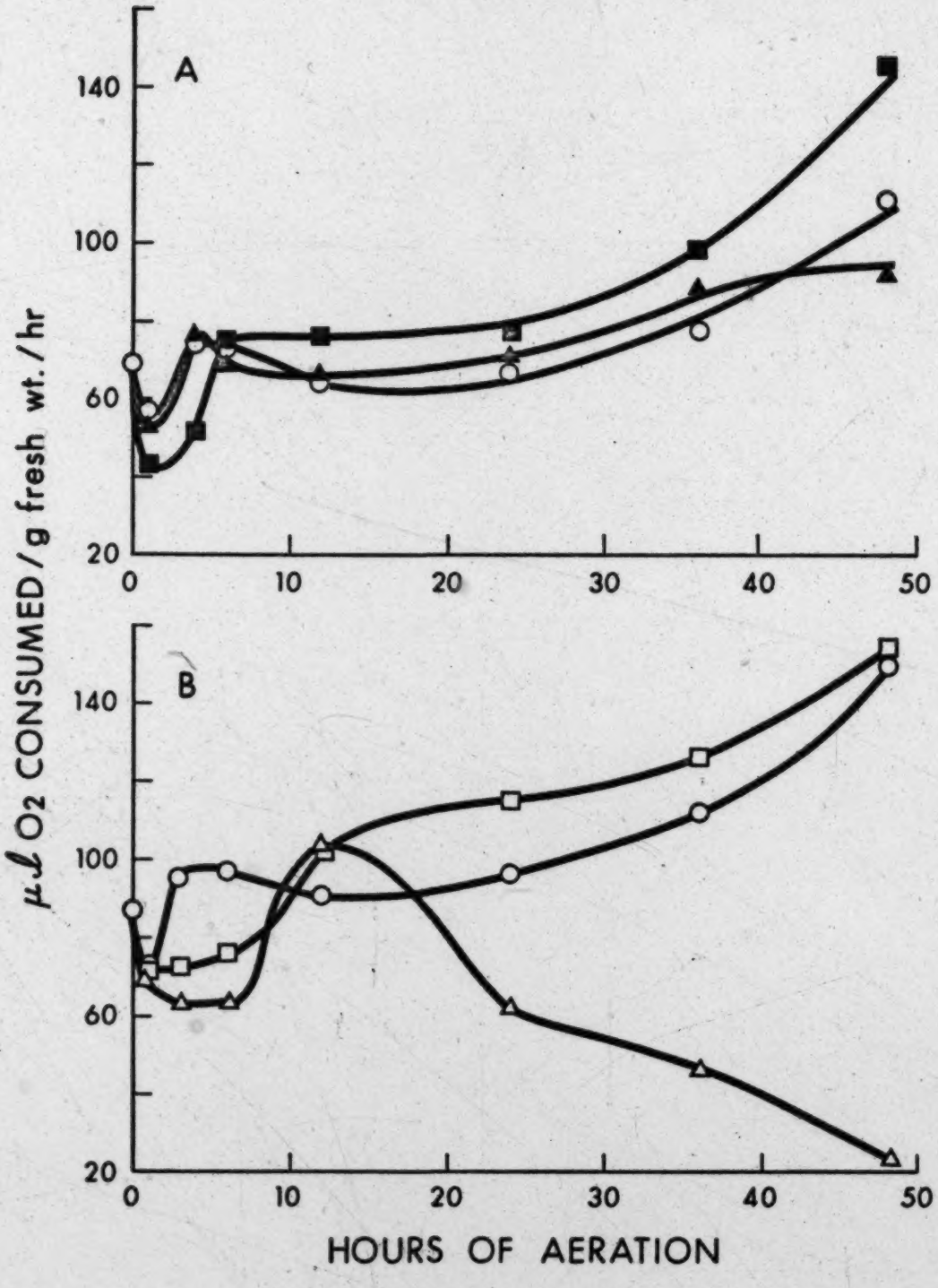
Disks aerated in GA_3 showed a higher induced respiration rate increase (about 15%) than the control disks (Fig. 4A). In methionine-treated disks the rise in respiration rate was similar to that of the control. Respiration rates, double that of the initial values, were found after 48 hr of aeration. Adams and Rowan (1970) have reported a doubling of respiration rates after 24 hr of aeration.

Although methionine is known to inhibit the respiration of turnip slices (Davies, 1966), this was not apparent for carrot slices (Fig. 4A). It was found, however, that ethionine, an analogue of methionine, clearly inhibited respiration of the disks after 20 hr of treatment (Fig. 4B).

Aminopterin, known for its inhibitory effect on the synthesis of THFA from dihydrofolate, did not have any major effect on the rate of induced respiration of aerated carrot slices (Fig. 4B); in fact, such disks actually showed a 10-20% stimulation of respiration over the control.

Fig. 4. *Effect of aeration on respiration of carrot tissue slices in the presence of different treatment solutions*

Disks (1 mm x 9 mm) were cut from greenhouse-grown carrots stored for 2 months at 4°C. Two replicates of 5 disks each were sampled at various times and the oxygen uptake determined by a YSI oxygen monitor. Fresh wt. of the disks was determined after an 8 min run. The mean values were plotted for carrot slices aerated in distilled water (control) (○), 0.1 mM GA_3 (■), 5 mM L-methionine (▲), 10 mM L-ethionine (△) and 0.02 mM aminopterin (□). Data for A and B are from two separate experiments.


A
B
140
100
60
20
0
10
20
30
40
50
μl O2 CONSUMED/g fresh wt./hr
HOURS OF AERATION

III. Studies of Amino Acid Levels during Storage and Changes during Aeration

A detailed assessment of the levels of free amino acids, particularly those associated with one-carbon metabolism, could clearly yield important data regarding the turnover of methyl groups during the enhanced biosynthetic activity which occurs during aeration of storage tissue slices. Before attempts were made to study the effect of aeration on amino acid levels of carrot slices, the effect of tissue source and storage conditions on initial levels was examined.

Amino acid analysis of carrot tissue stored for 2 months at 4°C revealed that the free amino acid pool consisted of three principal amino acids; 34% alanine, 29% glutamate and 23% serine (Table 3). Other amino acids detected were aspartate, valine, isoleucine, glycine and lysine. An approximately equal concentration of amino acids was released after hydrolysis of ethanol-insoluble protein extracted from these tissues.

In carrots stored for 6 months, the free amino acid levels showed an increase of over 300% while protein amino acids declined by 37% (Table 3). However, the total amino acids (free and protein) recovered from such tissues doubled during this additional storage period. It is clear from Table 3 that the almost 100 µmol/g fresh wt. increase in free amino acids cannot be attributed to amino acids liberated by protein degradation which amounts to only 14 µmol/g fresh wt. Serine showed the largest increase during storage. After 6 months of storage, free lysine was not detected but proline and methionine were now found. In general, all protein amino acids declined in concentration after 6 months' storage with the exception of methionine which doubled in concentration (Table 3).

Table 3. *Effect of storage at 4°C on free and protein amino acids of carrot tissue*

Mean values expressed as µmol/g fresh wt. tissue after duplicate analysis by Beckman 121 analyzer. F, free; P, protein; T, total; n.d., not detected; t, trace amounts.

Amino acid	Months of storage					
	2			6		
	F	P	T	F	P	T
Lys	0.19	2.19	*2.38*	n.d.	n.d.	*n.d.*
His	n.d.	0.72	*0.72*	n.d.	t	*t*
Arg	n.d.	0.56	*0.56*	n.d.	n.d.	*n.d.*
Asp	2.85	3.89	*6.74*	13.29	3.36	*16.65*
Thr	t	2.51	*2.51*	t	1.80	*1.80*
Ser	8.70	3.06	*11.76*	44.77	2.10	*46.87*
Glu	11.00	4.93	*15.93*	24.09	3.55	*27.64*
Pro	n.d.	2.29	*2.29*	4.02	1.86	*5.88*
Gly	0.23	3.92	*4.15*	0.98	3.47	*4.45*
Ala	13.20	4.02	*17.22*	42.68	3.12	*45.80*
Cys	n.d.	n.d.	*n.d.*	t	n.d.	*t*
Val	1.10	3.28	*4.38*	5.29	2.84	*8.13*
Met	n.d.	0.09	*0.09*	0.18	0.18	*0.36*
Ile	0.58	1.96	*2.54*	1.68	0.74	*2.42*
Leu	0.31	3.06	*3.37*	0.08	0.66	*0.74*
Tyr	n.d.	0.64	*0.64*	n.d.	n.d.	*n.d.*
Phe	n.d.	0.61	*0.61*	n.d.	n.d.	*n.d.*
Total	38.16	37.73	*75.89*	137.06	23.68	*160.74*

When changes in the principal free and protein amino acids were followed during aeration in distilled water (Table 4), it was observed that the total free amino acids declined from 38.16 µmol/g fresh wt. to 11.96 µmol/g fresh wt. by 48 hours. There was a general depletion of individual free amino acids and although not very pronounced in aspartate, the decline was dramatic in alanine. Total protein amino acids remained relatively unchanged during the early stages of aeration but by 48 hr of aeration displayed a 42% increase over the initial level. Although all protein amino acids showed an increase after aeration, on a percentage basis, glycine and methionine showed the most significant increase.

In connection with these analyses it was found that some amino acids were leached out of the slices during the incubation period. Table 5 summarizes the levels of the four major amino acids detected in the distilled water after various aerating periods. In this particular experiment the aerating medium was not changed. It is evident that the amino acids, with the exception of aspartate, re-entered the slices as the endogenous pools were depleted. Relatively high amounts of ammonia were also detected in the aerating solution together with trace amounts of glycine, valine and isoleucine. The amount of each amino acid leached out represented approximately 10% of the endogenous pool present before aeration.

Disks cut from 6-month stored tissue showed a decline in free amino acid levels from initially 137.06 µmol/g fresh wt. to 105.20 µmol/g fresh wt. after 24 hr of aeration (Table 6). In contrast to disks of 2-month stored tissue (Table 4), these disks show that levels of free serine did not decline during the aeration period studied (Table 6). Furthermore, several amino acids were not detected in either free or

Table 4. *Changes in free and protein amino acids during aeration of carrot slices*

Disks prepared from greenhouse-grown carrot tissue stored 2 months at 4°C. Mean values expressed as μmol/g fresh wt. tissue after duplicate analysis. F, free; P, protein; T, total; n.d., not detected; t, trace amounts.

Amino acid	Hours of aeration								
	0			24			48		
	F	P	T	F	P	T	F	P	T
Lys	0.19	2.19	*2.38*	0.05	1.78	*1.83*	0.02	2.82	*2.84*
His	n.d.	0.72	*0.72*	n.d.	0.59	*0.59*	n.d.	0.83	*0.83*
Arg	n.d.	0.56	*0.56*	n.d.	0.55	*0.55*	n.d.	0.83	*0.83*
Asp	2.85	3.89	*6.74*	2.58	4.23	*6.81*	1.99	5.68	*7.67*
Thr	t	2.51	*2.51*	t	2.50	*2.50*	n.d.	3.35	*3.35*
Ser	8.70	3.06	*11.76*	3.81	3.05	*6.86*	4.84	3.99	*8.83*
Glu	11.00	4.93	*15.93*	8.67	4.86	*13.53*	3.16	6.49	*9.65*
Pro	n.d.	2.29	*2.29*	n.d.	2.25	*2.25*	0.07	3.11	*3.18*
Gly	0.23	3.92	*4.15*	0.04	4.13	*4.17*	0.05	6.66	*6.71*
Ala	13.20	4.02	*17.22*	2.69	4.02	*6.71*	1.24	5.35	*6.59*
Cys	n.d.	n.d.	*n.d.*	n.d.	n.d.	*n.d.*	t	n.d.	t
Val	1.10	3.28	*4.38*	0.47	3.05	*3.52*	0.31	4.39	*4.70*
Met	n.d.	0.09	*0.09*	t	0.22	*0.22*	0.17	0.36	*0.53*
Ile	0.58	1.96	*2.54*	0.14	1.70	*1.84*	0.08	3.77	*3.85*
Leu	0.31	3.06	*3.37*	0.07	2.60	*2.67*	0.03	4.32	*4.35*
Tyr	n.d.	0.64	*0.64*	n.d.	0.51	*0.51*	n.d.	0.85	*0.85*
Phe	n.d.	0.61	*0.61*	n.d.	0.55	*0.55*	n.d.	0.89	*0.89*
Total	38.16	37.73	*75.89*	18.52	36.59	*55.11*	11.96	53.69	*65.65*

Table 5. *Leaching of amino acids from aerated carrot slices into distilled water during aeration*

Carrot tissue stored 2 months at 4°C. Results expressed as μmol/g fresh wt.

Amino acid	Hours of aeration			
	12	18	24	30
Asp	0.73	0.77	0.72	0.76
Ser	1.21	1.34	1.07	0.82
Glu	0.84	0.72	0.63	0.59
Ala	0.88	0.65	0.37	0.40
Total	3.66	3.48	2.79	2.57

protein form. The decline in the free amino acid pool was, furthermore, not comparable on a percentage basis to the decline shown by the previous tissues. This also emphasized the need for a uniform source of carrot tissue if meaningful comparisons between experiments are to be drawn.

The effect of GA_3 and methionine treatments on the free amino acid levels of aerated disks is shown in Table 7. In agreement with the data presented in Table 4, free amino acid levels declined during aeration of control disks. This also occurred in the GA_3 and methionine treatments. In the latter treatment, methionine taken up was subtracted from the total free amino acids recovered from the disks. Depletion of the free amino acid pools was most rapid and extensive in the GA_3 treatment; some amino acids disappeared entirely by 48 hr of aeration. An accumulation of methionine occurred in the methionine-treated disks, the levels of this

Table 6. *Changes in free and protein amino acids in aerated carrot tissue stored for 6 months at 4°C*

Results expressed as μmol/g fresh wt. tissue. F, free; P, protein; T, total; n.d., not detected; t, trace amounts.

Amino acid	Hours of aeration								
	0			12			24		
	F	P	T	F	P	T	F	P	T
Lys	n.d.	n.d.	n.d.	n.d.	t	t	n.d.	n.d.	n.d.
His	n.d.	t	t	n.d.	t	t	t	t	t
Arg	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Asp	13.29	3.36	16.65	14.65	3.07	17.72	11.01	3.11	14.12
Thr	t	1.80	1.80	t	1.60	1.60	n.d.	1.62	1.62
Ser	44.77	2.10	46.87	37.02	1.85	38.87	48.79	1.95	50.74
Glu	24.09	3.55	27.64	18.95	3.23	22.18	12.78	3.15	15.93
Pro	4.02	1.86	5.88	3.11	1.62	4.73	2.41	1.64	4.05
Gly	0.98	3.47	4.45	0.81	3.19	4.00	0.85	3.09	3.94
Ala	42.68	3.12	45.80	33.95	2.80	36.75	24.47	2.81	27.28
Cys	t	n.d.	t	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Val	5.29	2.84	8.13	4.26	2.42	6.68	3.39	2.34	5.73
Met	0.18	0.18	0.36	0.05	0.19	0.24	0.09	0.26	0.35
Ile	1.68	0.74	2.42	1.09	0.75	1.84	1.32	1.27	2.59
Leu	0.08	0.66	0.74	0.08	0.48	0.56	0.09	1.23	1.32
Tyr	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	t	n.d.	t
Phe	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	137.06	23.68	160.74	113.97	21.20	135.04	105.20	22.47	127.67

Table 7. *Changes in principal free amino acids during aeration of carrot tissue slices in different treatments*

Carrot tissue, greenhouse-grown, stored 2 months at 4°C. Results expressed as μmol/g fresh wt. tissue. Amino acids not listed were not detected. n.d., not detected; t, trace amounts.

Amino acid	Hours of aeration				
	0	12	24	36	48
Distilled water control					
Asp	3.72	3.94	4.44	2.28	1.80
Thr	0.51	0.38	0.59	0.44	0.23
Ser	3.85	2.25	3.79	3.26	3.10
Glu	2.61	3.31	5.29	3.16	2.53
Pro	0.26	t	t	t	t
Gly	0.13	0.13	0.18	0.09	0.13
Ala	5.08	2.09	2.39	0.84	0.43
Cys	0.09	t	0.16	t	t
Val	0.41	0.33	0.44	0.24	0.18
Met	0.05	n.d.	n.d.	n.d.	0.03
Ile	0.28	0.18	0.21	0.18	0.08
Leu	0.13	0.06	0.06	0.08	0.05
Total	17.12	12.67	17.54	10.57	8.56
0.1 mM GA_3					
Asp		2.10	1.85	1.72	0.84
Thr		0.56	0.36	0.28	0.13
Ser		3.35	3.33	4.36	2.57
Glu		2.51	2.39	2.10	1.05
Pro		t	t	t	t
Gly		0.11	0.08	0.07	0.05
Ala		2.98	1.18	0.69	0.18
Cys		t	t	t	n.d.
Val		0.43	0.29	0.23	0.08
Met		n.d.	n.d.	n.d.	n.d.
Ile		0.18	0.13	0.11	0.05
Leu		t	t	t	t
Total		12.22	9.61	9.56	4.95
5 mM L-methionine					
Asp		2.72	2.31	2.36	1.79
Thr		0.64	0.39	0.36	0.28
Ser		6.74	5.64	5.54	5.15
Glu		2.10	1.67	1.66	1.23
Pro		t	n.d.	n.d.	n.d.
Gly		0.15	0.16	0.13	0.10
Ala		3.03	2.00	1.34	0.67
Cys		t	n.d.	n.d.	t
Val		0.49	0.39	0.31	0.21
Met		3.33	4.53	5.43	7.02
Ile		0.29	0.25	0.18	0.20
Leu		0.18	t	t	t
Total		19.57	17.34	17.31	16.65
Total - methionine		16.24	12.81	11.88	9.63

Table 8. *Changes in free and protein amino acids in carrot disks in relation to treatment after 36 hr of aeration*

Carrot tissue, greenhouse-grown, stored 2 months at 4°C. Results expressed as μmol/g fresh wt. tissue. F, free; P, protein; T, total; n.d., not detected; t, trace amounts.

Amino acid	0 Time			36 hr aeration								
				Distilled water control			0.1 mM GA_3			5 mM L-methionine		
	F	P	T	F	P	T	F	P	T	F	P	T
Lys	t	1.28	*1.28*	t	1.55	*1.55*	t	1.37	*1.37*	t	1.55	*1.55*
His	n.d.	0.41	*0.41*	n.d.	0.50	*0.50*	n.d.	0.49	*0.49*	n.d.	0.54	*0.54*
Arg	n.d.	0.71	*0.71*	n.d.	0.77	*0.77*	n.d.	0.77	*0.77*	n.d.	0.86	*0.86*
Asp	3.72	1.91	*5.63*	2.28	1.94	*4.22*	1.72	2.52	*4.24*	2.36	2.26	*4.62*
Thr	0.51	1.03	*1.54*	0.44	1.01	*1.45*	0.28	1.09	*1.37*	0.36	1.19	*1.55*
Ser	3.85	1.28	*5.13*	3.26	1.32	*4.58*	4.36	1.35	*5.71*	5.54	1.51	*7.05*
Glu	2.61	1.92	*4.53*	3.16	1.88	*5.04*	2.10	1.97	*4.07*	1.66	2.17	*3.83*
Pro	0.26	0.87	*1.13*	t	1.05	*1.05*	t	0.96	*0.96*	n.d.	1.04	*1.04*
Gly	0.13	1.76	*1.89*	0.09	1.73	*1.82*	0.07	1.77	*1.84*	0.13	1.93	*2.06*
Ala	5.08	1.70	*6.78*	0.84	1.67	*2.51*	0.69	1.71	*2.40*	1.34	1.89	*3.23*
Cys	0.09	t	*0.09*	t	t	*t*	t	t	*t*	n.d.	t	*t*
Val	0.41	1.39	*1.80*	0.24	1.32	*1.56*	0.23	1.37	*1.60*	0.31	1.48	*1.79*
Met	0.05	0.10	*0.15*	n.d.	0.07	*0.07*	n.d.	0.04	*0.04*	5.43	0.03	*5.46*
Ile	0.28	0.91	*1.09*	0.18	0.91	*1.09*	0.11	0.89	*1.00*	0.18	1.03	*1.21*
Leu	0.13	1.61	*1.74*	0.08	1.56	*1.64*	t	1.58	*1.58*	t	1.78	*1.78*
Tyr	n.d.	0.36	*0.36*	n.d.	0.42	*0.42*	n.d.	0.32	*0.32*	n.d.	0.46	*0.46*
Phe	n.d.	0.36	*0.36*	n.d.	0.66	*0.66*	n.d.	0.54	*0.54*	n.d.	0.71	*0.71*
Total	17.12	17.60	*34.72*	10.57	18.36	*28.93*	9.56	18.74	*28.30*	17.31	20.43	*37.74*
										11.88†		*32.31†*

† Figure represents total free amino acids less methionine accumulated from the medium

amino acid increasing 140 fold by 48 hr of aeration. In comparison to the other treatments, such disks also maintained a significantly higher level of free serine throughout the aeration period.

Total protein amino acids, for the 36 hr study period selected, increased slightly in both the control and the GA_3 treatment (Table 8). A greater increase in protein amino acids was observed in the methionine-treated disks where most of the acids listed showed increases over the control values.

Estimations of total water-soluble protein were also carried out (Table 9). In the distilled water control synthesis of soluble proteins occurred during the aeration. The results are not as clear-cut in other treatments although by 48 hr of aeration all of the treatments showed a marked increase in the amount of extractable water-soluble protein.

Table 9. *Effect of treatment and aeration on the quantity of water-soluble protein extracted from carrot slices*

Mean value of duplicate estimates expressed as mg soluble protein extracted/g fresh wt. tissue.

Treatment	Hours of aeration				
	0	12	24	36	48
Distilled water control	1.81	2.05	1.96	2.14	2.21
0.1 mM GA_3		1.39	2.05	2.04	2.09
5.0 mM L-methionine		2.20	1.82	1.95	2.44
10.0 mM L-ethionine		2.04	1.94	1.92	2.41
0.02 mM aminopterin		1.69	2.07	2.00	2.77

IV. Folate Derivatives in Carrot Tissue Slices and Changes during Aeration

Carrot tissues examined for folates by Toepfer *et al.* (1951) were found to contain detectable levels of materials which promoted the growth of the folate assay organisms, *L. casei* and *S. faecalis*. To obtain more information regarding the nature and levels of folate pool constituents, as well as possible changes occurring during aeration and treatment, the following detailed investigation of carrot disks was undertaken. Firstly, the principal folate derivatives were identified in extracts of freshly cut slices followed by an analysis of the changes occurring during aeration of slices in each of the different treatments used previously.

The results, presented in Fig. 5 and Table 10, establish, for the first time, the nature of the folate derivatives present in carrot root tissue. Fig. 5 is a typical elution sequence of the derivatives extracted from tissues stored for 2 months at 4°C. Compounds present in the first two major peaks (Fig. 5, peaks a and b) gave growth promoting properties typical of formyl derivatives and were eluted at positions corresponding to 10-formylTHFA and 10-formylTHFA diglutamate, respectively, as established by the criteria of Roos and Cossins (1971). The large peak (Fig. 5, peak c) coincided with authentic 5-methylTHFA. The minor peaks (Fig. 5, peaks d and e) probably represent THFA and 5-methylTHFA diglutamate, respectively, as identified by Roos and Cossins (1971), also occur in small quantities in pea tissues.

When carrot tissue slices were aerated in the different media (Fig. 6) it was found that the levels of 'free' folates increased dramatically in the control (distilled water), GA_3, methionine and ethionine

Fig. 5. *Chromatography of folate derivatives extracted from freshly-cut carrot tissue slices*

Analysis of greenhouse-grown carrot tissue stored 2 months at 4°C. After DEAE-cellulose chromatography, the fractions were assayed for folate compounds using *L. casei*. The derivatives shown are: a, 10-formylTHFA; b, 10-formylTHFA diglutamate; c, 5-methylTHFA; d, THFA; e, 5-methylTHFA diglutamate. *S. faecalis* gave positive growth responses to the compounds in peaks a, b and d but not in c.

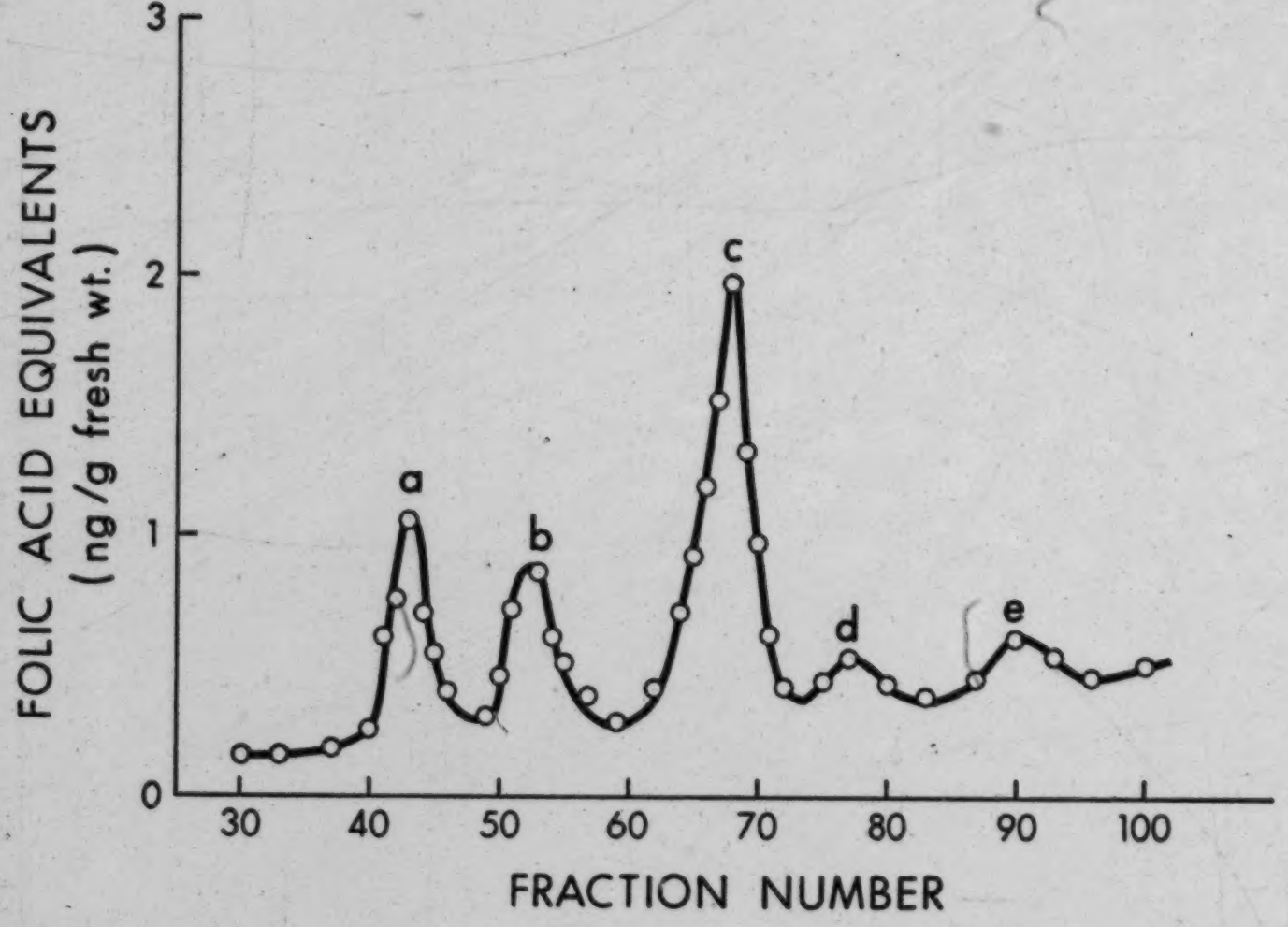
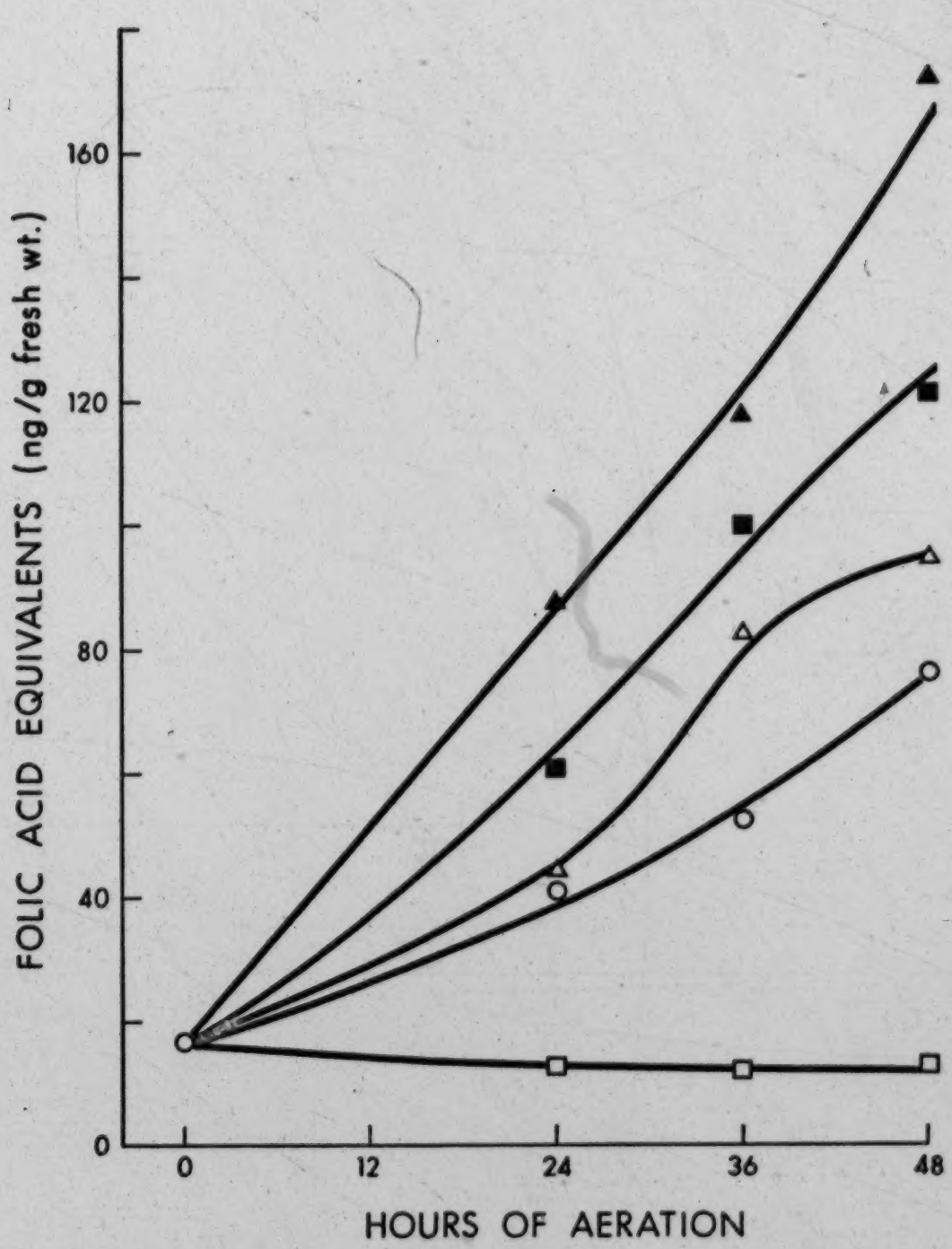

FOLIC ACID EQUIVALENTS
(ng/g fresh wt.)
3
2
1
0
a
b
c
d
e
30
40
50
60
70
80
90
100
FRACTION NUMBER

Fig. 6. *Effect of aeration and treatment on levels of 'free' folates in carrot tissue slices*

Cumulative figures from Table 10 are represented following estimation using *L. casei*. Greenhouse-grown carrot tissue stored 2 months at 4°C was sliced for the following treatments: distilled water (○), 0.1 mM GA_3 (■), 5.0 mM L-methionine (▲), 10.0 mM L-ethionine (△) and 0.02 mM aminopterin (□).


FOLIC ACID EQUIVALENTS (ng/g fresh wt.)
160
120
80
40
0
0
12
24
36
48
HOURS OF AERATION

treatments but declined in the aminopterin treatment. These increases were considerably greater in disks aerated in GA_3 and methionine as compared to the control. The depletion of the tetrahydrofolate pool as a result of treatment with aminopterin was as expected.

Column chromatography of the 'free' folates in slices aerated over a 48 hr period in the five treatments gave the results summarized in Fig. 7. A comparison of Figs. 5 and 7 emphasizes the magnitude of the increases in the individual folate derivatives as a result of aeration under the various treatment conditions. The principal derivative, 5-methyl-THFA (Fig. 7, peak c), showed greatest accumulation in disks aerated in the presence of methionine. For sake of clarity, the elution pattern for the ethionine-treated slices is not shown in Fig. 7 as it would be essentially superimposed on the GA_3 values. Aminopterin-treated disks show a marked depletion of 10-formylTHFA diglutamate and 5-methylTHFA (Fig. 7, peaks b and c, respectively).

Analysis of slices from the various treatments at 24 and 36 hr of aeration gave levels intermediate to those of Figs. 5 and 7 and are summarized in Table 10. The major derivative found in this tissue, 5-methylTHFA, accounted for 61% of the mono and diglutamate derivatives present at the time of initial slicing. This proportion increased to 75% after 48 hr of aeration in the distilled water control. Table 10 also indicates that formylated mono and diglutamate derivatives accumulated during aeration of the slices in distilled water but proportionately not as much as 5-methylTHFA. A similar pattern is also evident for slices aerated in GA_3, L-methionine and L-ethionine. An accumulation of 5-methyl-THFA in the presence of exogenous methionine is contrary to what occurs

Fig. 7. *Chromatography of folate derivatives from carrot slices aerated for 48 hr in different treatment solutions*

After DEAE-cellulose chromatography, the fractions were assayed for folate compounds using *L. casei*. The derivatives shown are: a, 10-formylTHFA; b, 10-formylTHFA diglutamate; c, 5-methylTHFA. Greenhouse-grown carrot tissue stored 2 months at 4°C was sliced for the following treatments: distilled water (○), 0.1 mM GA_3 (■), 5.0 mM L-methionine (▲) and 0.02 mM aminopterin (□).

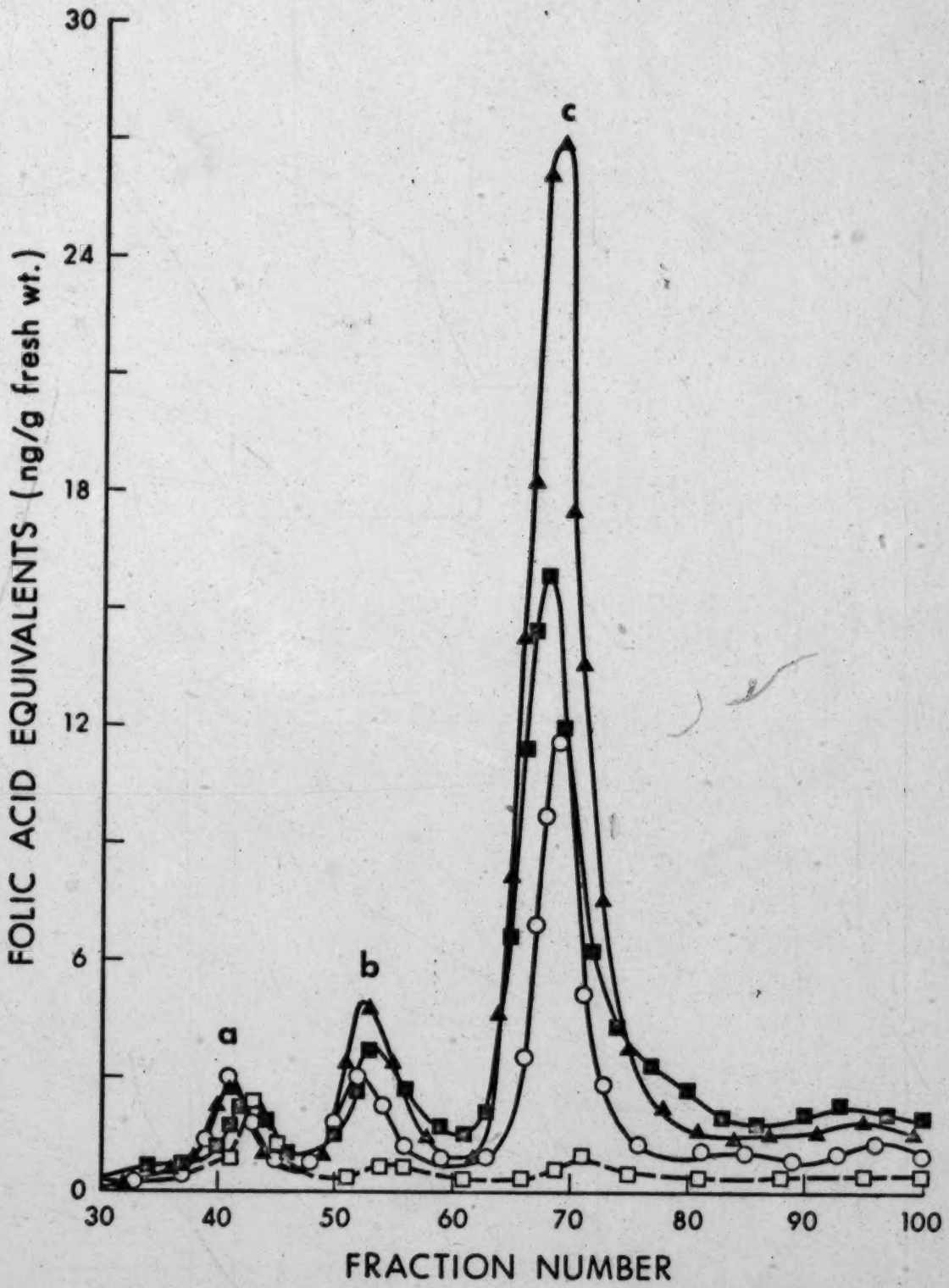

30
24
18
12
6
0
FOLIC ACID EQUIVALENTS (ng/g fresh wt.)
a
b
c
30
40
50
60
70
80
90
100
FRACTION NUMBER

Table 10. *Effect of aeration and treatment on levels of 'free' THFA derivatives in carrot tissue slices*

Greenhouse-grown tissue stored 2 months at 4°C used for slicing. Results expressed as folic acid equivalents (ng/g fresh wt.).

Treatment	Hours of aeration			
	0	24	36	48
10-HCO-THFA				
Distilled water control	3.70	4.28	7.91	9.60
0.1 mM GA_3		5.92	9.24	6.20
5.0 mM L-methionine		6.51	7.41	8.60
10.0 mM L-ethionine		7.04	8.56	5.40
0.02 mM aminopterin		5.54	6.18	7.08
10-HCO-THFA diglutamate				
Distilled water control	3.07	6.36	8.32	10.70
0.1 mM GA_3		5.16	16.20	14.24
5.0 mM L-methionine		11.86	13.08	23.37
10.0 mM L-ethionine		6.02	6.07	5.25
0.02 mM aminopterin		6.71	2.45	0.99
5-CH_3-THFA				
Distilled water control	10.12	30.42	36.33	57.68
0.1 mM GA_3		49.88	75.43	101.60
5.0 mM L-methionine		70.29	96.57	141.58
10.0 mM L-ethionine		32.72	68.69	85.00
0.02 mM aminopterin		1.47	3.67	4.64

under such conditions in yeast (Lor and Cossins, 1972) and in rat liver (Buehring *et al.*, 1972). The levels of monoglutamate derivatives in GA_3, methionine and ethionine-treated disks were higher than those in the distilled water control throughout the aeration period (Table 10). After 36 hr of aeration, 10-formylTHFA declined in both the GA_3 and the ethionine-treated slices.

V. Polyglutamyl Folate Derivatives of Carrot Tissue Slices

Earlier work in this laboratory as well as preliminary work in this study indicated that polyglutamyl folate derivatives exist to varying degrees in many higher plant tissues including carrot. In order to examine the changes in such polyglutamates in carrot slices aerated in different treatment solutions, a series of extracts were prepared from slices aerated in distilled water (control), GA_3, methionine and ethionine solutions. Polyglutamyl folates cannot be directly assayed microbiologically, but can be assayed after hydrolysis to 'free' folates by treatment with γ-glutamyl carboxypeptidase. The peptidase used in this study was prepared from germinating pea cotyledons (Roos and Cossins, 1971) and hydrolyzes polyglutamyl folates to monoglutamates. Since the carrot tissue used in this study (Fig. 8 and 9) was from a different harvest than that used for experiments represented in Table 10, it will be noted that the level of 'free' folates (before conjugase treatment) was higher at initial slicing (42 ng/g fresh wt., Fig. 8, control) than the value shown in Table 10 (16.89 ng/g fresh wt.).

The control treatment (Fig. 8, control) shows that 75% of the folates in carrot tissue, at the time of initial slicing, existed as polyglutamates. A trend of this magnitude appeared to be maintained throughout

Fig. 8. *Levels of folates in extracts from carrot tissue slices aerated under different treatment conditions*

Greenhouse-grown carrot tissue stored 2 months at 4°C was used for slicing. Aliquots of extracts were assayed using *L. casei*. Hydrolysis of polyglutamyl derivatives was achieved by incubation of the extracts with pea cotyledon γ-glutamyl carboxypeptidase. The data is presented as total levels after carboxypeptidase treatment (□), levels before carboxypeptidase treatment or 'free' folates (■), levels of polyglutamyl derivatives (▨).

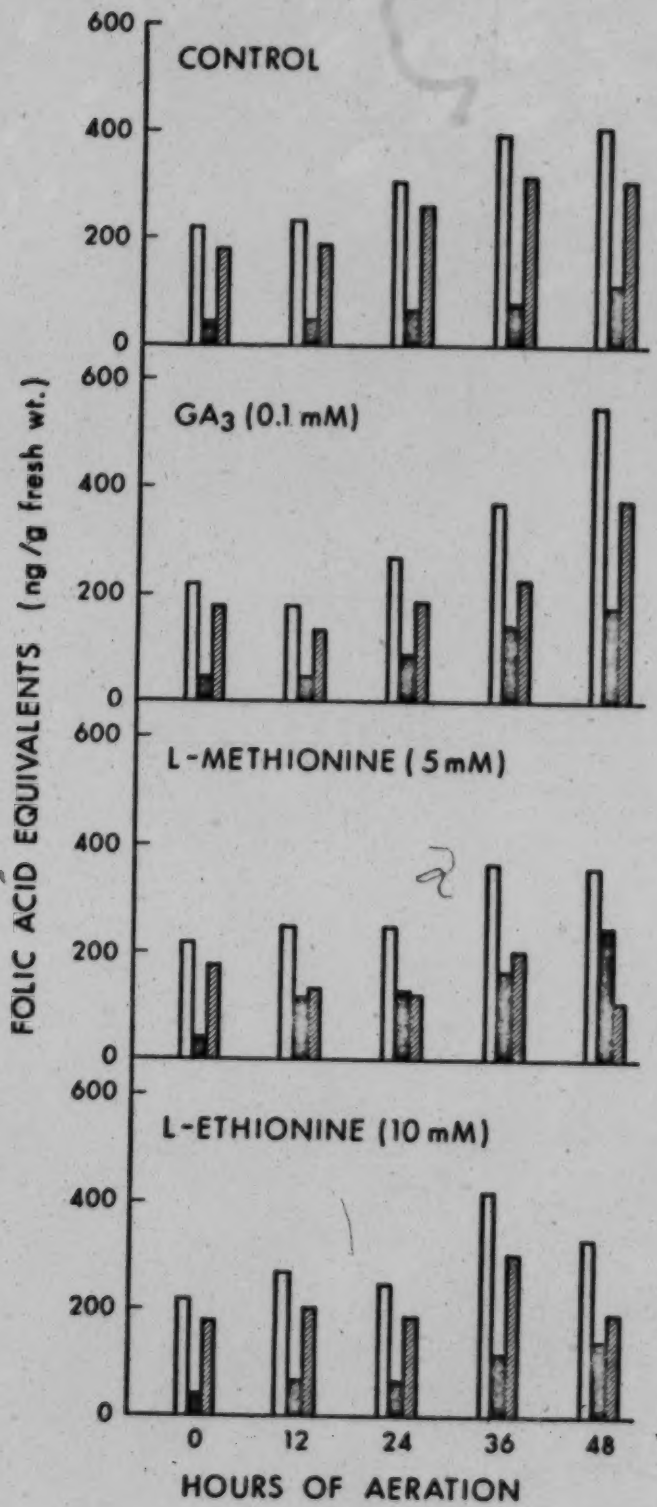
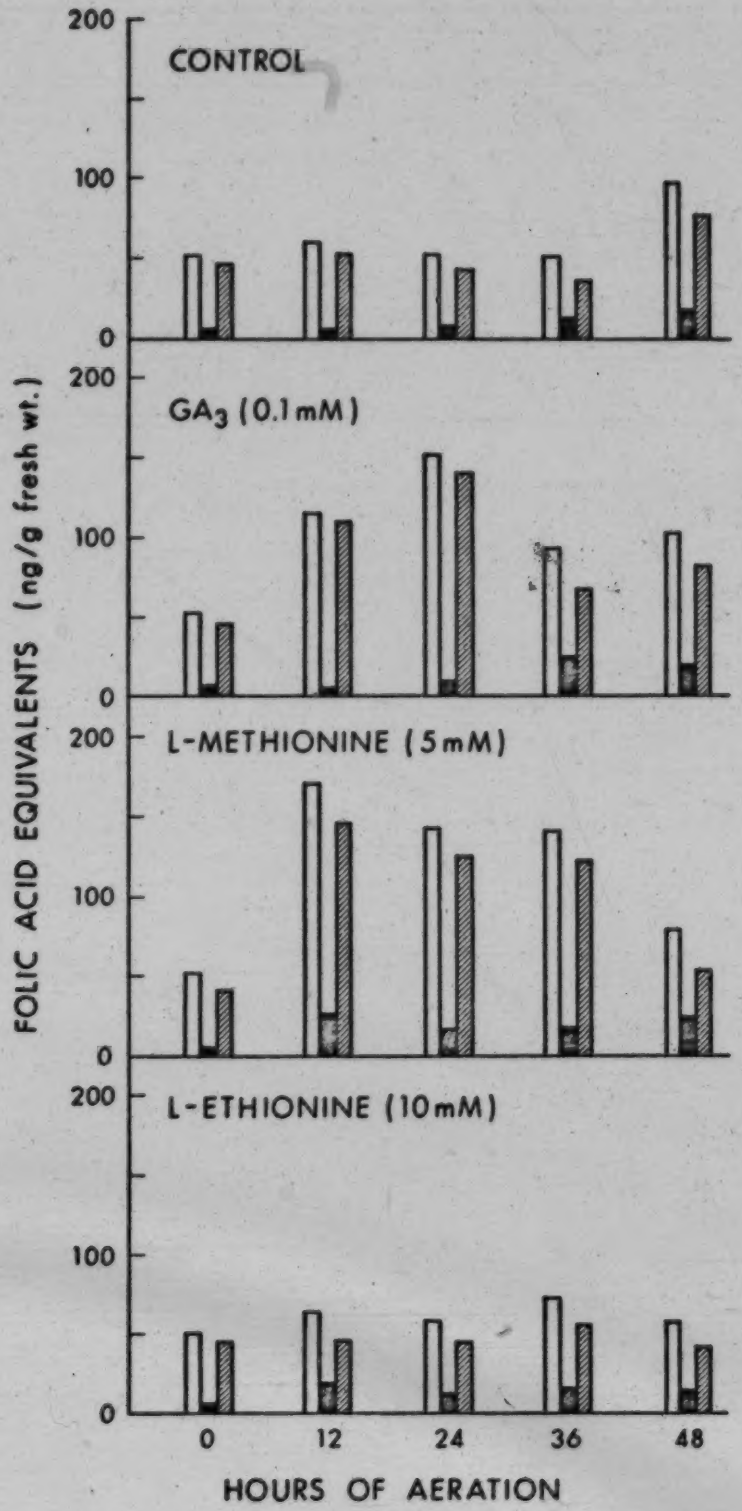

CONTROL
GA3 (0.1 mM)
L-METHIONINE (5 mM)
L-ETHIONINE (10 mM)
600
400
200
0
FOLIC ACID EQUIVALENTS (ng/g fresh wt.)
0
12
24
36
48
HOURS OF AERATION

Fig. 9. *Levels of formylated and unsubstituted folates in extracts from carrot tissue slices aerated under different treatment conditions*

Greenhouse-grown carrot tissue stored 2 months at 4°C was used for slicing. Aliquots of extracts were assayed using *S. faecalis*. Hydrolysis of polyglutamyl derivatives was achieved by incubation of the extracts with pea cotyledon γ-glutamyl carboxypeptidase. The data is presented as total levels after carboxypeptidase treatment (□), levels before carboxypeptidase treatment (■), levels of polyglutamyl derivatives (▨).


CONTROL
GA3 (0.1mM)
L-METHIONINE (5mM)
L-ETHIONINE (10mM)
200
100
0
FOLIC ACID EQUIVALENTS (ng/g fresh wt.)
0
12
24
36
48
HOURS OF AERATION

the period of aeration in distilled water. Consequently, increases in 'free' folate derivatives were observed as a direct result of aeration.

Carrot slices aerated in GA_3 for 48 hr showed the highest levels of total folates after conjugase treatment (Fig. 8). Disks aerated in methionine showed a lower rate of polyglutamate accumulation than that observed in the control. There was, however, a corresponding increase in monoglutamate forms which may suggest that polyglutamate forms were being hydrolyzed during the aeration period by endogenous carboxypeptidases. Disks aerated in ethionine showed a pattern similar to that of methionine. The trends of (a) the accumulation of 'free' folates during the aeration process and (b) the 'free' folate levels in methionine, GA_3 and ethionine treatments (Fig. 8) being greater than the control, are in keeping with trends established in Table 10.

Using *S. faecalis*, which grows in the presence of formylated and unsubstituted folate derivatives, an estimate of these compounds was made (Fig. 9). Approximately 6 ng/g fresh wt. folic acid equivalents were determined as formylated derivatives at initial slicing (Fig. 9). A small increase in these derivatives before conjugase treatment can be seen (Fig. 9, control) and is in agreement with Table 10. The concentration of formylated polyglutamates appeared to remain fairly constant in the control until the later stages of aeration. In GA_3 and methionine-treated slices, however, formylated polyglutamates increased significantly (Fig. 9) during the early stages of aeration and then declined. Comparing these two treatments (Fig. 9, GA_3 and methionine) to corresponding treatments in Fig. 8, it is probable that a major portion of the polyglutamate pool during the intermediate stages of aeration in the GA_3 and methionine

treatments existed as formyl derivatives rather than as methyl derivatives. This situation appeared to be reversed in the control disks.

VI. Enzymes of One-Carbon Metabolism: Activities as a Function of Aeration and Treatment

The activity and regulation of enzymes of C_1 metabolism have now been investigated in greater detail since their presence was first established in carrot tissue (Fedec and Cossins, 1972). In the present studies it was established that carrot tissue slices contain the following enzymes of C_1 metabolism: 5,10-methyleneTHFA reductase, 5-methylTHFA:homocysteine transmethylase, serine hydroxymethyltransferase and 10-formylTHFA synthetase. In the majority of cases the specific activity of these enzymes was significantly enhanced by GA_3 treatment of the tissues.

5,10-Methylenetetrahydrofolate reductase

In a study of 5,10-methyleneTHFA reductase (Table 11) it was found that the specific activity of this enzyme increased during aeration of the disks in distilled water. By 12 hr, the specific enzyme activity had increased by 124% over the zero-time value but after 48 hr it had declined to well below the initial value.

Like the results of the control, disks aerated in GA_3 showed maximal 5,10-methyleneTHFA reductase activity by 12 hr of aeration (173% of the zero-time value). These GA_3-aerated disks displayed the highest activity of all treatments for the 12 and 24 hr assay periods. The decline of activity in GA_3-treated slices during later stages of aeration was not as rapid as in the control. Disks treated with L-methionine also showed enhanced reductase activity but in this treatment it was lower during the

Table 11. *Effect of aeration on 5,10-methyleneTHFA reductase activity of treated carrot slices*

The complete reaction system contained, in 0.32 ml: 10 µmol of K-phosphate, buffer (pH 6.4), 5 nmol of FAD, 5 nmol of menadione, 5 µmol of formaldehyde, 0.1 µCi of 5-[methyl-^{14}C]methylTHFA (1 µCi/16 nmol) and cell-free extract from carrot slices. Results are expressed as nmol product formed/mg protein/hr at 30°C. All assays were performed in duplicate.

Treatment	Hours of aeration				
	0	12	24	36	48
Distilled water control	1.38	3.19	2.69	1.37	0.74
0.1 mM GA_3		3.76	2.78	2.41	1.22
5.0 mM L-methionine		2.71	2.44	2.50	2.12
10.0 mM L-ethionine		2.53	2.37	2.17	1.89
0.02 mM aminopterin		2.83	1.43	0.82	0.40

early hours of aeration and showed little decline after 48 hr. Extracts of L-ethionine-treated disks gave results similar to those observed for the methionine treatment but showed a more extensive loss of enzyme activity by 48 hr.

5-Methyltetrahydrofolate:homocysteine transmethylase

During aeration, the specific activity of 5-methylTHFA:homocysteine transmethylase also varied considerably in each of the treatments employed (Table 12). In the distilled water control, enzyme activity was maximal after 24 hr of aeration at which time it was 33% greater than the initial zero-time value. After 48 hr of aeration, however, the activity had declined markedly.

Table 12. *Effect of aeration on 5-methylTHFA:homocysteine transmethylase activity of treated carrot slices*

The complete reaction system contained, in 0.5 ml: 50 µmol of K-phosphate buffer (pH 6.3), 1 µmol of L-homocysteine freshly prepared from the thiolactone form, 0.1 µCi of 5-[methyl-^{14}C]methylTHFA (1 µCi/16 nmol) and cell-free extract from carrot slices. Results are expressed as pmol product formed/mg protein/hr at 30°C. All assays were performed in duplicate.

Treatment	Hours of aeration				
	0	12	24	36	48
Distilled water control	152.4	148.8	202.2	132.0	65.4
0.1 mM GA_3		255.5	280.8	427.8	215.4
5.0 mM L-methionine		159.6	196.8	228.0	247.2
10.0 mM L-ethionine		229.8	76.2	199.8	237.0
0.02 mM aminopterin		147.6	94.8	54.6	27.0

Disks aerated in GA_3 showed the most dramatic increase in transmethylase activity. In this treatment maximal activity occurred after 36 hr of aeration. At this time, a 182% increase in activity over the initial zero-time value was apparent. This treatment also maintained the highest activity of all treatments during 12-36 hr of aeration, in fact, at 36 and 48 hr the activity extracted from the GA_3-treated slices was triple that of the control disks. Methionine-treated disks, on the other hand, showed a gradual increase in transmethylase activity over the entire 48 hr aeration period with an activity double that of the control at 36 hr and nearly quadruple at 48 hr of aeration. Disks treated with ethionine did not behave like those of methionine. Rather, there appeared to be a dramatic decrease in activity at 24 hr followed by some increase during the remainder of the aeration period. Aminopterin-treated disks showed a continuous

decline in transmethylase activity throughout the aeration period. This trend is in keeping with the observation that the synthesis of 5-methylTHFA was also considerably reduced by this treatment (Table 10).

Serine hydroxymethyltransferase

In general the activity of serine hydroxymethyltransferase showed increases during aeration under the treatment conditions employed (Table 13). Following 12 hr of aeration in distilled water, the activity doubled and remained fairly high, notwithstanding minor fluctuations. Treatment with GA_3 enhanced the activity of this enzyme during the aeration period to a greater degree; in fact, the general trend in this treatment appeared to indicate that serine hydroxymethyltransferase activity increased over the aeration period. In methionine-treated slices, the enzyme appeared to reach maximal activity at 12 hr and then decline to lower levels equal to or lower than those of the control.

Table 13. *Effect of aeration on serine hydroxymethyltransferase activity of treated carrot slices*

The complete reaction system contained, in 0.4 ml: 30 µmol of K-phosphate buffer (pH 8.0), 0.1 µmol of pyridoxal 5-phosphate, 1.6 µmol of THFA, 2 µmol of 2-mercaptoethanol, 0.1 µCi of L-[3-^{14}C]serine (1 µCi/17.7 nmol) and Sephadex G-15 desalted cell-free extract from carrot slices. Results are expressed as nmol product formed/mg protein/hr at 30°C. All assays were performed in duplicate.

Treatment	Hours of aeration				
	0	12	24	36	48
Distilled water control	0.98	1.99	1.63	1.66	1.93
0.1 mM GA_3		2.19	2.85	2.30	3.60
5.0 mM L-methionine		2.00	1.97	1.65	1.65

10-Formyltetrahydrofolate synthetase

Both crude and desalted extracts were compared for activity of 10-formylTHFA synthetase (Table 14). In desalted extracts, it was found that with aeration there was, as a general rule, an increase in enzyme activity in most of the treatments. With the exception of the 36 hr value, GA_3-treated slices showed a greater synthetase capacity than did control disks. An overall increase in specific activity of 60 to 70% was observed over the 48 hr aeration period. Methionine-treated disks did not appear to have as marked an increase in synthetase activity as the other two treatments.

Table 14. *Effect of aeration on 10-formylTHFA synthetase activity in treated carrot slices*

The complete reaction system contained, in 0.8 ml: 10 µmol of triethanolamine-HCl buffer (pH 8.0), 150 µmol of TRIS-formate buffer (pH 8.0), 2.5 µmol of $MgCl_2$, 200 µmol of KCl, 4 µmol of THFA, 2 µmol of ATP, 10 µmol of 2-mercaptoethanol and either Sephadex G-15 desalted or nondesalted cell-free extract from carrot slices. Results are expressed as nmol product formed/mg protein/hr at 30°C. All assays were performed in duplicate.

Treatment	Hours of aeration				
	0	12	24	36	48
Crude cell-free extracts:					
Distilled water control	918	1296	1464	1548	1854
0.1 mM GA_3		1242	1308	1626	1902
5.0 mM L-methionine		1206	1302	1146	972
Desalted cell-free extracts:					
Distilled water control	1005	1167	1429	1740	1632
0.1 mM GA_3		1350	1614	1416	1770
5.0 mM L-methionine		1128	1272	1290	1494

Crude tissue extracts which had not been desalted showed similar trends for the three treatments studied but the difference in enzyme activity of disks aerated in methionine was more pronounced in these preparations when compared to the control, especially after 48 hr where the activity from methionine-treated disks was half that seen in the control and was very similar to the initial zero-time value.

VII. Effects of L-Methionine and Other Compounds on the Activity of C_1 Enzymes *in vitro*

Results presented earlier have shown that L-methionine-treated carrot slices not only accumulated 5-CH_3-THFA but showed enhanced activity *in vivo* of several enzymes of one-carbon metabolism. This is in contrast to the effect methionine normally exhibits in microorganisms and other tissues. By inhibition or repression, methionine plays a significant role in the regulation of certain enzymes of C_1 metabolism in microorganisms, animal tissues and higher plants (Table 1, p. 27). Two such enzymes are 5,10-methyleneTHFA reductase and 5-methylTHFA:homocysteine transmethylase. SAM may also inhibit the reductase (Table 1). It was, therefore, considered worthwhile to investigate the suggestion that methionine may have little effect *in vitro* in regulating these enzymes in aerated carrot slices. The study was also extended to include other directly-related enzymes and effects of SAM on the two enzymes previously mentioned. Effects of methionine as related to physiological concentrations found in carrot tissue were the prime target for observation.

5,10-MethyleneTHFA reductase

The effect of several concentrations of L-methionine on the activity of 5,10-methyleneTHFA reductase is shown in Table 15. At the

concentrations studied, methionine inhibited the activity of this enzyme by 20 to 30%. Most inhibition occurred at very low levels, i.e., physiological levels. It has already been shown that very low or only trace amounts of methionine were found in aerated carrot tissue slices (Table 4) and in intact carrots (Table 3).

Table 15. *Effect of L-methionine on 5,10-methyleneTHFA reductase activity* in vitro

Reaction conditions were identical to those of Table 11 except that variable quantities of L-methionine were added to the reaction system as indicated.

L-methionine (Final concn., mM)	Enzyme activity (% of control)
0	100
0.01	67.8
0.05	66.2
0.1	65.9
0.5	69.1
1	70.7
6	77.7
10	81.4

It is interesting to note in this connection that published data for plants have shown no effect of methionine *in vitro* on 5,10-methylene-THFA reductase activity in pea cotyledon (Dodd and Cossins, 1970) or pea root-tip extracts (Cossins *et al.*, 1972). This enzyme is strongly inhibited by methionine in yeast (Lor and Cossins, 1972) and by SAM in rat liver (Kutzbach and Stockstad, 1971).

Several concentrations of SAM were used in order to determine whether this compound would have any inhibitory effect on the activity of 5,10-methyleneTHFA reductase (Table 16). No appreciable inhibition was observed at the concentrations tested.

Table 16. *Effect of S-adenosyl methionine on 5,10-methyleneTHFA reductase activity* in vitro

Reaction conditions were identical to those of Table 11 except that variable quantities of SAM were added to the reaction system as indicated.

SAM (Final concn., mM)	Enzyme activity (% of control)
0	100
1	9[illegible].4
2	108.5
5	107.4

5-MethylTHFA:homocysteine transmethylase

The effect of several concentrations of L-methionine on the activity of 5-methylTHFA:homocysteine transmethylase is shown in Table 17 using a crude cell-free extract prepared from carrot disks aerated for 24 hr at which time the specific activity of this enzyme in distilled water was maximal. At physiological concentrations there was no apparent effect on the activity of the transmethylase enzyme, while at the highest concentrations the activity was only inhibited by about 11%. Although L-methionine does not appear to have an effect *in vitro* on 5-methylTHFA: homocysteine transmethylase from carrot tissue slices, this enzyme is inhibited by methionine in yeast (Lor and Cossins, 1972) and pea tissue extracts (Cossins *et al.*, 1972).

Table 17. *Effect of L-methionine on 5-methylTHFA:homocysteine transmethylase activity* in vitro

Reaction conditions were identical to those of Table 12 except that variable quantities of L-methionine were added to the reaction system as indicated.

L-methionine (Final concn., mM)	Enzyme activity (% of control)
0	100
0.01	102.5
0.05	102.0
0.1	101.2
0.5	101.5
1	93.8
6	92.7
10	89.2

Several concentrations of SAM appeared to show a stimulatory or activating effect on the activity of 5-methylTHFA:homocysteine transmethylase *in vitro* (Table 18).

Table 18. *Effect of S-adenosyl methionine on 5-methylTHFA:homocysteine transmethylase activity* in vitro

Reaction conditions were identical to those of Table 12 except that variable quantities of SAM were added to the reaction system as indicated.

SAM (Final concn., mM)	Enzyme activity (% of control)
0	100
1	139.1
2	136.7
5	163.8

Serine hydroxymethyltransferase

During assay of crude cell-free extracts for serine hydroxymethyltransferase activity, it was observed that very little loss of activity occurred after storage for 24 hr at 3°C. When the extract was desalted through Sephadex G-15, serine hydroxymethyltransferase activity decreased from 11.3 nmol/mg protein/hr immediately after desalting to 4.6 nmol/mg protein/hr after 2 hr of storage at 3°C. Therefore, desalted extracts were used immediately. The effect of L-methionine on the activity of serine hydroxymethyltransferase is shown in Table 19. At the concentrations used in this study, it appears that methionine had little or no effect on the activity of this enzyme *in vitro*.

Table 19. *Effect of L-methionine on serine hydroxymethyltransferase activity* in vitro

Reactions conditions were identical to those of Table 13 except that variable quantities of L-methionine were added to the reaction system as indicated.

L-methionine (Final concn., mM)	Enzyme activity (% of control)
0	100
0.05	108
0.5	107
5	110

10-FormylTHFA synthetase

Despite reports to the contrary (Iwai *et al.*, 1967; Rabinowitz and Pricer, 1963) it was found that, in the assay of 10-formylTHFA synthetase, 2 µmol of THFA per reaction mixture for assay were sub-optimal

(Table 20). Experimentation showed that 4 µmol of THFA as outlined by Hiatt (1965) gave near optimal activities without significantly adding to the expense of this reagent.

Table 20. *Effect of THFA concentration on the activity of 10-formylTHFA synthetase*

Reaction conditions were identical to those of Table 14 except that the quantity of THFA was varied according to the concentrations listed below. Sephadex G-15 desalted cell-free extract was used. Results are expressed as nmol product formed/mg protein/hr at 30°C.

THFA concentration µmol/reaction	Specific enzyme activity
2	337
4	422
6	432
8	350

Using physiological concentrations of methionine likely to be found in carrot tissue, no apparent inhibition of 10-formylTHFA synthetase was observed (Table 21). However, at concentrations of 1.0 mM methionine, such as would have accumulated in the disks after 36 to 48 hr of aeration in L-methionine, strong inhibition of the enzyme was observed (Table 21). It should also be pointed in this regard that decreased activity *in vivo*, compared to the control, was shown in methionine-treated disks after 24 to 48 hr of aeration (Table 14).

Since some SAM synthetase activity is likely to occur in these crude extracts, increases in methionine concentration added to the reaction mixture, together with a fixed amount of ATP, could result in decreased

Table 21. *Effect of L-methionine on 10-formylTHFA synthetase activity* in vitro

Reaction conditions were identical to those of Table 14 except that variable quantities of L-methionine were added to the reaction system as indicated. Sephadex G-15 desalted cell-free extract was used.

L-methionine (Final concn., mM)	Enzyme activity (% of control)
0	100
0.01	102.3
0.05	104.5
0.1	65.2
1.0	32.6

availability of ATP for the 10-formylTHFA synthetase reaction. Reduced activity of the latter enzyme did not appear to be borne out by [^{14}C]-formate incorporation studies in methionine-treated carrot slices, as will be shown in the following section.

VIII. Incorporation of [^{14}C]Formate in Aerated Carrot Tissue Slices

Since it has been established in the present work that methionine and GA_3 had considerable effect on the biosynthesis of folate derivatives, further investigation to elucidate whether such treatments altered the ability of the tissue to metabolize a one-carbon precursor was undertaken. Incorporation of ^{14}C from formate was followed into free and protein amino acids of preaerated carrot slices with attention focussed on amino acids closely related to C_1 metabolism, for example, serine, glycine and methionine.

After carrot slices were preaerated in the different treatment solutions, samples of 25 disks were incubated for 1 hr in [^{14}C]formate. Extracts were then prepared, separated into constituent fractions and the radioactivity of each fraction determined (Table 22). A high proportion of the label (65 to 73%) appeared as $^{14}CO_2$ in each of the treatments.

One of the main features shown in Table 22 is that maximum incorporation of [^{14}C]formate into the various fractions occurred after approximately 36 hr of aeration for the control and the methionine treatment. In GA_3-treated disks maximal labelling occurred after approximately 24 hr. Considerably more label appeared in the lipid fraction of GA_3-treated disks than in any of the other treatments. Likewise, GA_3-treated slices showed the highest label in the 6 M amino acid fraction, indicative of label in SAM. The organic acid fraction showed most label in the control. The largest amount of label in the 4 M amino acid fraction appeared in disks aerated in methionine while disks aerated in GA_3 showed the least incorporation into this fraction of the three treatments.

Labelling of protein amino acids was slightly lower in the GA_3-treated slices than in the control (Table 23). On a percentage basis, however, the GA_3-treated disks had a higher percent incorporation of ^{14}C into protein. Methionine-treated disks, on the other hand, showed little ^{14}C incorporation into protein amino acids.

Paper chromatography of the labelled free amino acids (Table 24) showed that ^{14}C was concentrated in only a few of the free amino acids present in slices. Serine was the most heavily labelled amino acid in the control and GA_3-treated disks while methionine was the most heavily labelled amino acid in the disks aerated in L-methionine.

Table 22. *Metabolism of [^{14}C]formate by preaerated carrot tissue slices*

Carrot disks were preaerated in the three treatment solutions. 25 disks from each aeration period and treatment were incubated in a closed 25 ml Warburg flask containing 1 µmol [^{14}C]formate (2.5 µCi/µmol; pH 5.9) in 4 ml distilled water for 1 hr at 30°C. Results are expressed as c.p.m./ 25 disks extracted.

Time and treatment	Fraction					
					Amino acids	
	CO_2	Lipids	Sugars	Organic acids	4 M	6 M
0 hr						
Distilled water control	184400	390	5000	40200	48100	1700
12 hr						
Distilled water control	422000	2800	13200	85600	109000	3900
0.1 mM GA_3	436600	2700	11600	82000	71300	5200
5 mM L-methionine	330300	120	6100	48600	22[illegible]00	6800
24 hr						
Distilled water control	394700	2600	23000	123000	102200	4400
0.1 mM GA_3	734500	6700	21800	92000	66900	8100
5 mM L-methionine	227400	130	2900	28200	134000	4600
36 hr						
Distilled water control	1005100	1900	5700	130800	126500	5400
0.1 mM GA_3	742200	11000	9100	75400	83700	11700
5 mM L-methionine	535500	180	11000	54800	202900	8300
48 hr						
Distilled water control	266300	1300	9800	153300	86600	5400
0.1 mM GA_3	527700	12700	13400	70000	72800	10200
5 mM L-methionine	544800	210	17100	51100	175800	4000

Table 23. *Incorporation of [^{14}C]formate into various fractions by carrot disks aerated for 36 hr*

Incubation conditions identical to those given in Table 22. Results are expressed as c.p.m./25 disks extracted.

Fraction	Treatment					
	Distilled water control		0.1 mM GA_3		5 mM L-methionine	
	c.p.m.	% of total	c.p.m.	% of total	c.p.m.	% of total
CO_2	1005100	73.3	742200	73.3	535500	65.3
Lipids	1900	0.1	11000	1.1	180	0.02
Sugars	5700	0.4	9100	0.9	11000	1.3
Organic acids	130800	10.0	75400	7.4	54800	6.7
Amino acids						
4 M	126500	9.3	83700	8.2	202900	24.7
6 M	5400	0.4	11700	1.2	8300	1.0
Protein amino acids	89000	6.6	79400	7.8	7200	0.9
Total	1364400		1012500		819880	

Table 24. *Incorporation of [^{14}C]formate into free amino acids by aerated carrot tissue slices*

Incubation conditions identical to those given in Table 22. Results are expressed as c.p.m./25 disks extracted.

Time and treatment	Amino acid					
	Asp	Ser	Glu	Gly	Ala	Met
0 hr						
Distilled water control	5300	13600	2200	800	1500	3300
12 hr						
Distilled water control	3500	33500	4200	1200	2300	4500
0.1 mM GA_3	1860	26400	2140	1430	2000	4800
5 mM L-methionine	2650	35600	2200	1150	2900	72700
24 hr						
Distilled water control	6900	26600	8100	3700	2700	3300
0.1 mM GA_3	1210	22000	2500	2400	1640	3600
5 mM L-methionine	1000	25400	1930	1400	2280	56200
36 hr						
Distilled water control	5500	40200	8000	2800	3600	3500
0.1 mM GA_3	3000	25200	4000	1600	2120	2900
5 mM L-methionine	2700	33800	3900	2800	2900	95200
48 hr						
Distilled water control	4300	18100	8600	650	2560	2800
0.1 mM GA_3	2040	18200	4300	2200	2850	2300
5 mM L-methionine	1700	31100	2900	2500	2600	82400

More detailed analysis of the labelled amino acids, using the Beckman amino acid analyzer equipped with stream division, showed that most of the neutral and acidic amino acids contained ^{14}C to some degree in disks which had been preaerated for 36 hr (Table 25). About 67% of the total ^{14}C incorporated into free amino acids was found in serine in control and GA_3-treated slices. The remainder of the label appeared in aspartate and glutamate. Disks aerated in methionine showed only 31% of the ^{14}C label in serine while methionine accounted for 57% of the ^{14}C.

When the specific activity of the free amino acids was determined, it was clear that aerated slices in all treatments had an enhanced ability to incorporate the C_1 unit from formate (Table 26). This serves to show that the major products of metabolism were indeed amino acids closely related to one-carbon metabolism, namely, methionine, serine and glycine. The specific radioactivities of individual amino acids were not equally affected by the treatments. For example, the activity of these three amino acids was less in the methionine-treated slices than in control slices preaerated for 36 hr. On the other hand, in GA_3-treated slices, the specific activity of methionine was considerably higher than found in the other treatments.

In the protein amino acids, from 36 hr aerated carrot slices, the major amino acid labelled in the control and GA_3 treatment was serine (Table 27); accounting for 66 and 71% of the label, respectively. This was followed by aspartate having 12 and 13% of the ^{14}C in respective treatments. On the other hand, slices aerated in methionine showed a much larger proportion for the label in glycine, constituting 29% of the total. This was followed by aspartate (23%) and serine (19%). All of

Table 25. *Incorporation of [^{14}C]formate into free amino acids by carrot disks aerated for 36 hr*

Incubation conditions identical to those given in Table 22. Results, following detailed stream division analysis, are expressed as c.p.m./25 disks extracted. n.d., not detectable.

Amino acid	Treatment					
	Distilled water control		0.1 mM GA_3		5 mM L-methionine	
	c.p.m.	% of total	c.p.m.	% of total	c.p.m.	% of total
Met sulfoxide	4480	3.4	2580	3.3	530	0.3
Asp	11930	8.9	8210	10.5	12310	6.4
Thr	1750	1.3	1440	1.8	380	0.2
Ser	88920	66.8	52290	66.9	58600	30.6
Glu	16720	12.5	6990	8.9	4030	2.1
Pro	1220	0.9	2280	2.9	1220	0.6
Gly	2660	1.9	300	0.4	2050	1.1
Ala	4330	3.3	300	0.4	840	0.4
Cys	300	0.2	380	0.5	150	0.1
Val	n.d.	-	1060	1.4	1600	0.8
Met	840	0.6	1750	2.2	109140	56.9
Ile	n.d.	-	600	0.8	760	0.4
Total	133150		78180		191610	

Table 26. *Specific activity in free amino acids after [^{14}C]formate feeding of carrot slices*

Freshly-cut or preaerated carrot disks were incubated according to the conditions given in Table 22. Results, following detailed stream division analysis, are expressed as c.p.m./μmol amino acid.

Amino acid	Treatment			
	Distilled water control		0.1 mM GA_3	5 mM L-methionine
	Hours of aeration			
	0	36	36	36
Met sulfoxide	-	11800	5800	1892
Asp	4310	10230	9720	8550
Ser	11530	44680	19660	17340
Glu	2830	8660	5460	3990
Gly	19370	44300	7500	26000
Ala	930	8490	810	1020
Met	13330	84000	175000	32900

the other amino acids shown were labelled, in the three treatments, to about the same degree. It is clear that the total ^{14}C incorporated from [^{14}C]formate into the protein amino acids was very low in disks aerated in methionine.

When the specific activities of the protein amino acids were intercompared, it was observed that slices aerated in distilled water and GA_3 showed a greater incorporation of labelled amino acids into protein (Table 28). It is interesting to note that, in preaerated slices, the specific activity of the protein amino acids was less in GA_3-treated disks

Table 27. *Incorporation of [^{14}C]formate into protein amino acids by carrot disks aerated for 36 hr*

Incubation conditions identical to those given in Table 22. Results, following detailed stream division analysis, are expressed as c.p.m./25 disks extracted. n.d., not detectable.

Amino acid	Treatment					
	Distilled water control		0.1 mM GA_3		5 mM L-methionine	
	c.p.m.	% of total	c.p.m.	% of total	c.p.m.	% of total
Met sulfoxide	1090	1.6	n.d.	-	n.d.	-
Asp	7730	11.6	6660	13.2	1150	23.2
Thr	140	0.2	340	0.7	90	1.8
Ser	44210	66.3	35570	70.7	960	19.3
Glu	1530	2.3	1070	2.1	400	7.9
Pro	140	0.2	270	0.5	50	0.9
Gly	3830	2.3	1660	3.3	1410	28.5
Ala	700	1.1	840	1.7	50	0.9
Cys	750	1.1	590	1.2	200	3.9
Val	300	0.4	340	0.7	140	2.7
Met	5470	8.2	2670	5.3	240	4.9
Ile	480	0.7	200	0.4	170	3.4
Leu	230	0.3	70	0.1	110	2.1
Total	66600		50280		4970	

Table 28. *Specific activity in protein amino acids after [^{14}C]formate feeding of carrot slices*

Freshly-cut or preaerated carrot disks were incubated according to the conditions given in Table 22. Results, following detailed stream division analysis, are expressed as c.p.m./μmol amino acid.

Amino acid	Treatment			
	Distilled water control		0.1 mM GA_3	5 mM L-methionine
	Hours of aeration			
	0	36	36	36
Asp	3090	6550	4350	830
Ser	26820	54580	43380	1040
Glu	570	1330	890	300
Gly	1690	3650	1540	1200
Ala	360	690	810	50
Met	38230	124300	102600	13300

than in control disks. The methionine-treated slices, however, showed values that were consistently lower. Methionine, an end product of C_1 metabolism, showed the highest specific activity in all cases.

DISCUSSION

I. Preliminary Considerations of Tissue, Composition and Changes Occurring during Aeration

In studies which involve large quantities of plant tissue over extended periods, as in this particular investigation, a major prerequisite for critical work is that a uniform source of tissue must be maintained. Aging of storage tissue disks, related measurements of respiration and changing constituents have, in the past, been generally made using material which has been 'purchased locally'. In the present work, attention was paid not only to possible variation of such tissues but standardization was sought towards uniformity of material, aeration technique and control of bacterial contamination.

To facilitate comparison of published data on storage tissue disks with results to be gained regarding one-carbon metabolism of such a system, the choice of tissue was also of some importance. Carrot tissues have not only been used extensively, but slices of uniform size (1 mm x 9 mm) are easy to prepare in bulk. Furthermore, if aseptic conditions are employed during slicing, carrot tissues appear to be less susceptible to bacterial contamination than other common storage tissues (Cossins and Blawacky, unpublished data). Of further advantage, carrot slices do not show meristematic activity during aeration (MacDonald *et al.*, 1961) and slices thicker than 0.55 mm do not initiate vessel element formation (Mizuno *et al.*, 1971). For these reasons, it was rational to conclude that minimal growth, differentiation and bacterial contamination would occur during aeration of carrot slices for 48 hr. At the same time this

higher plant system, being relatively simple physiologically, had potential for studies of C_1 metabolism with the ease of handling and manipulation approaching that of microbial systems.

Since the major emphasis in this work was a study of folate synthesis and its possible regulation, it was important initially to determine the extent of bacterial contamination during aeration and to control it. Bacteria, having capacities for C_1 metabolism far exceeding those of plant tissues, could clearly contribute folate derivatives to plant tissue extracts in undetermined amounts. A second factor that had to be considered was possible use of bacteriostatic compounds. These were not employed in this study because of possible interference in the growth response of the assay organisms used in the analysis of folates. Thirdly, the common bacteriostat, chloramphenicol, although used by Leaver and Edelman (1965*b*), has more recently been shown to have adverse effects on this system, causing depression of cell expansion (Setterfield, 1970), inhibition of protein synthesis (Jacoby and Sutcliffe, 1962; Ap Rees and Bryant, 1971), inhibition of RNA synthesis and respiration (Bryant and Ap Rees, 1971). Clearly, to avoid or minimize the external factors interfering with the metabolic processes enhanced by aeration, aseptic treatment of the slices was the most logical approach.

Routine employment of aseptic techniques and microporous filtering of solutions combined with frequent media changes decreased bacterial counts (Table 2) to values lower than those observed in previous studies of storage tissue disks (Edelman and Hall, 1965; Reed and Kolattukudy, 1966). In this regard, it was found that compounds, such as methionine, ethionine and GA_3, added to the aerating solutions were a major source

of the contaminating bacteria and it would therefore appear that microporous filtering or sterilization should be a standard prerequisite in the handling of such experimental systems.

Periods of cold storage drastically changed the amino acid composition of harvested carrots (Table 3). Clearly, the response to aeration of tissues stored under unspecified conditions would obviously be affected. This aspect was assessed by observing the respiration of three different groups of carrot slices; greenhouse-grown either fresh or stored and those 'locally purchased'. Fig. 3 indicates that basal respiration of carrot slices varies with the tissue source. This rate is known to be dependent, to a degree, on maturity as well as storage. For example, Rosenstock *et al.* (1971) observed high respiration rates in slices cut from young potato tubers but lower rates when mature tubers were used. These workers also observed that the respiration actually declined upon aeration of the younger slices, therefore, the respiration curve of the fresh greenhouse material (Fig. 3) may in part be due to the age of this particular material. Disks cut from greenhouse-grown carrots stored for 2 months at 4°C gave a basal respiration rate similar to that of the locally purchased carrots (Fig. 3). This value, 88 µl O_2 consumed/g fresh wt., was intermediate to values previously reported for freshly cut carrot disks, e.g., 48 µl O_2/g fresh wt./hr (Goh and Wiskich, 1967) and 108 µl O_2/g fresh wt./hr (Ap Rees and Royston, 1971).

After 24 hr of aeration the respiration rate of carrot slices has been shown to increase by as much as 100% (Adams and Rowan, 1970) and as little as 35% by Ap Rees and Royston (1971). Fig. 3 shows that an 88% increase in respiration rate was observed in 'locally purchased' carrot

tissue slices whereas only an 11% increase was observed in a 24 hr aeration period for slices cut from greenhouse grown and stored material. From this study and from the values given in the literature, it is clear that the locally bought carrot tissues vary widely with respect to respiratory rates. Considerable variations in respiration rate even occur in different mature roots of a single cultivar (Adams, 1970*b*). Such variation has been ascribed to differences in the endogenous levels of ADP.

Respiration rates on a per gram fresh wt. basis are also known to be affected by slice thickness. For example, in potato slices, Davies (1960) found rates to be inversely proportional to slice thickness up to 3.0 mm. For these reasons, slices of uniform thickness were used in the present work.

The decline in respiratory rate which immediately followed slicing (Fig. 3; 4A, B) has been reported by Adams (1970*a*) but not by other workers in this field. Although this phenomenon has, so far, not been explained satisfactorily, it may be related to ADP/ATP ratios which are known to change dramatically as mitochondrial numbers increase (Verleur *et al.*, 1970).

Unlike turnip storage disks (Davies, 1966), methionine did not inhibit respiration of the carrot slices used in this study (Fig. 4A). Progressive inhibition was observed, however, in the presence of L-ethionine (Fig. 4B), an observation also made by Atkinson and Polya (1968), indicating that sensitivity to this methionine analogue developed as aeration progressed.

Slices aerated in GA_3 showed an enhanced respiration rate of about 15% over the control (Fig. 4A). This rise was higher than that

observed in Palmer's (1966) study of beet disks where 0.1 to 0.01 mM GA_3 enhanced respiration by approximately 7%. Since increased respiration would conceivably be dependent upon synthesis of RNA and protein (Ap Rees and Royston, 1971), these enhanced rates may be interpreted as evidence for not only the synthesis of protein occurring during aeration but also a stimulation of such synthesis by GA_3.

In the analysis of free amino acids; serine, alanine, glutamate and aspartate accounted for the major part of free amino acid pool in the tissue initially (Table 3). These results are consistent with those of Alabran and Mabrouk (1973) but are not in agreement with those of Otsuka and Take (1969) or Bourke *et al.* (1967). The latter two groups found substantial quantities of threonine and valine and decreased amounts of aspartate. Total free amino acid content of carrot in this study was, furthermore, found to vary between 38 µmol/g fresh wt. (Tables 3 and 4) and 17 µmol/g fresh wt. (Table 7), values which are 5 to 10 times higher than those reported for carrot tissue by Bourke *et al.* (1967). Such differences are, on the whole, greater than those expected from variation in variety, climate, soil fertilization, cultivation, harvesting and storage effects as claimed by Alabran and Mabrouk (1973). In support of this contention, Lee (1973) showed that only a two-fold increase in free amino acids of beetroot tissue after application of more than recommended amounts of nitrate fertilizer could be expected. Table 3 also shows that the total free amino acids increased by 360% in carrots stored 6 months. Proline and methionine appeared where these had been lacking initially. This indicates, then, that protein degradation may be occurring and further that such degradation may be partially responsible for the large release of amino acids during storage. Since the initial amino acid

estimation was made on carrot tissue already stored for 2 months, it is quite possible that the greater quantity of total free amino acids observed in this study in comparison with the quantity observed by Bourke *et al.* (1967), who used locally purchased material, may be due to protein degradation already begun.

During aeration of carrot disks, the free amino acid levels declined (Tables 4, 6, 7), the specific activities of key enzymes of C_1 metabolism increased (Tables 11-14), the concentration of folate derivatives increased (Fig. 6, Table 10) and the levels of protein amino acids rose (Table 4). Depletion of the free amino acid pools during aeration was not entirely accounted for by increases in the protein amino acids (Table 4). A small part of the difference could, however, be accounted for by amino acids lost to the aerating solution (Table 5). Although no previous reports of depletion of amino acid pools in aerated carrot slices have been made, it is characteristic of other species (e.g., MacDonald *et al.*, 1961; Thurman *et al.*, 1963) where an accompanying increase in protein synthesis occurs.

In the present work, synthesis of new protein during aeration was indicated by the increased pools of the protein amino acids (Tables 4, 8) and by the increased amounts of water-soluble protein extracted from aerated slices (Table 9). It should be noted that the increases in soluble protein shown by slices treated with methionine and ethionine (Table 9) appear to be contrary to the findings of Trewavas (1972) and Stekol (1963) who suggested that these two compounds inhibited protein synthesis in higher plant tissue. This data (Table 9) is further contradicted by the fact that, during aeration of carrot slices in methionine followed by [^{14}C]formate

feeding, little ^{14}C appeared in protein amino acids of this treatment as compared to the distilled water control (Table 27). , It must be remembered, however, that total free amino acids decreased during the aeration of slices in methionine (Table 7). Further investigation will be necessary to clarify the possible effect of methionine on protein synthesis in this system.

II. Folate Synthesis during Aeration of Carrot Tissue Slices

Although estimates of folic acid equivalents have previously been made in carrot tissues (Toepfer *et al.*, 1951), the present investigation is the first to identify the individual derivatives in carrot tissue and to study enzymes associated with their synthesis and interconversion. In agreement with previous reports on folate derivatives in higher plant tissues (e.g., Santini *et al.*, 1964; Batra *et al.*, 1973; Cossins and Shah, 1972; Chan *et al.*, 1973) the major derivative found in carrot tissue was 5-CH_3-THFA; representing about 61% of the mono and diglutamate forms or 'free' folates present (Fig. 5, Table 10). Also present in the tissue were lesser amounts of 10-HCO-THFA mono and diglutamates. During aeration these derivatives, particularly 5-CH_3-THFA, increased. Depletion of the 'free' pool of folate derivatives was observed only in disks aerated in the presence of aminopterin. Judging from the rapid depletion of derivatives in this treatment, it can be concluded that an active turnover of 5-CH_3-THFA must occur during aeration.

When the effect of exogenous L-methionine on carrot folates is considered (Fig. 6, Table 10), it is clear that this system is unlike yeast (Lor and Cossins, 1972) or rat liver (Buehring *et al.*, 1972). Instead of L-methionine causing a depletion of the 5-CH_3-THFA pool, an extensive

accumulation was observed (Table 10; Fig. 6 and 7). Since methionine is known to repress methionine adenosyl transferase in yeast (Ferro and Spence, 1973) as well as enzymes responsible for synthesis of homocysteine, it is possible that the methionine-treated disks accumulated 5-CH_3-THFA for the following main reasons: (a) methionine is not extensively converted to SAM because the necessary enzyme may be repressed, hence an accumulation of 5-CH_3-THFA would result, and (b) a lack of available homocysteine as an acceptor for the methyl group of 5-CH_3-THFA may limit its utilization. The latter proposal was implied as a possible explanation for increased methylated derivatives observed in rust uredospores (Jackson *et al.*, 1970). Certainly the synthesis of methionine would not be controlled solely by 5-CH_3-THFA:homocysteine transmethylase as this enzyme was neither repressed (Table 12) nor inhibited (Table 17) to any degree by L-methionine, the end product of this reaction.

Although Toepfer *et al.* (1951) and Freed (1966) have published folate data for fresh carrot tissue, it should be pointed out that the analysis actually failed to yield as much information as claimed. For example, assays with *S. faecalis* were designated 'free folates', while assay with *L. casei* gave values termed 'total folates'. Since *S. faecalis* does not respond to methylated derivatives (Blakley, 1969) it is incorrect to consider such results 'free folates' when in reality this term includes all derivatives which are not conjugated. Comparatively speaking, the present investigation showed 6.2 ng/g fresh wt. folic acid equivalents in freshly sliced carrot tissue (Fig. 9) as assayed by *S. faecalis* whereas Toepfer *et al.* (1951) reported values in the range of 13-49 ng/g fresh wt. The present study also found 42.1 ng/g fresh wt. folic acid equivalents

in freshly sliced carrot tissue (Fig. 8) using *L. casei* compared to 46-76 ng/g fresh wt. as reported by Toepfer *et al.* (1951). Obviously a good deal of variability is possible between tissues and methods. More important, however, Fig. 8 and 9 demonstrate that a major proportion of the folate derivatives (75%) in carrot tissue exist as polyglutamates. This proportion was essentially maintained when carrot slices were aerated in distilled water.

In general, synthesis of conjugated and unconjugated folates occurred in all treatments during aeration of carrot slices (Fig. 8 and 9). This had not previously been studied in aerated storage tissue disks of any species but such a rapid increase in folate concentration must be related to the increases in protein synthesis, enzyme activity and respiration that occur during aeration.

The higher levels of 'free' folates observed in GA_3, methionine and ethionine-treated slices (Fig. 8, before conjugase treatments) agree with elevated quantities of derivatives observed in detailed column chromatographic analysis (Fig. 7, Table 10). In L-methionine-treated disks (Fig. 8), however, the levels after conjugase treatment are similar after the 36 and 48 hr aeration periods, although an increase in 'free' folates (before conjugase treatment) occurred. Since it has been suggested that glutamyl residues may be added prior to formation of the 5-CH_3-THFA derivative (Sengupta and Cossins, 1971), these results may indicate an increased carboxypeptidase activity cleaving the polyglutamate reserve rather than an increase in the synthesis of 5-CH_3-THFA monoglutamate. This aspect remains to be investigated.

Fig. 9 shows that 6.2 ng/g fresh wt. folic acid equivalents (assayed by *S. faecalis*) represented essentially formylated derivatives. This figure compared favorably with the figure of 6.77 ng/g fresh wt. obtained by column chromatography (Table 10). Such a close correlation verifies that, in carrot slices, formylated derivatives existed mainly in the N^{10} position and little, if any, were to be found in the N^5 position. This was further substantiated by the fact that *P. cerivisiae* failed to respond when used to assay fractions 60-68 (Fig. 5), a position in the elution sequence characteristic of N^5-formylTHFA (Roos and Cossins, 1971).

After conjugase treatment of the zero-time control extract, 25% of the total folates were found to be formyl derivatives (compare Fig. 8 and 9). Aeration increased the total folate level considerably by 48 hr (Fig. 9) but the proportion of formyl derivatives remained the same indicating a continuity of the derivatives with respect to substitution. The results for the methionine-treated disks were different, however, in that after 48 hr of aeration 59% of the polyglutamates were formylated. Such elevated levels in 10-HCO-THFA polyglutamates were shown to occur in rat liver slices treated with L-methionine (Buehring *et al.*, 1972). While carrot slices show a similarity in this respect to such treatment, they differ markedly from rat liver in their ability to accumulate large quantities of 5-CH_3-THFA (Table 10) whereas depletion occurs in liver tissue.

III. Regulation of C_1 Metabolism and Related Enzyme Studies

The presence of an active C_1 metabolism in aerated carrot disks was verified by the presence and subsequent increased activity of the following key enzymes: 5,10-methyleneTHFA reductase, 5-methylTHFA:

homocysteine transmethylase, serine hydroxymethyltransferase and 10-formyl-THFA synthetase (Tables 11-14). Such increased activity is consistent with increased synthesis of 5-CH_3-THFA and 10-HCO-THFA observed in this system (Fig. 7, Table 10). Furthermore, incorporation of ^{14}C from [^{14}C]-formate leading to high specific activities in methionine, serine and to a lesser extent glycine, particularly after aeration of the disks (Table 26), is indicative of the extent to which enzyme activation occurred in some of the treatments (e.g., distilled water, GA_3, L-methionine).

On the basis of results presented in this thesis, it is reasonable to propose a scheme representing possible control mechanisms in the carrot slice system which could regulate the flow of one-carbon units through these folate-mediated pathways (Fig. 10).

Regulation of methyl group biogenesis appears to centre on 5,10-CH_2-THFA reductase and 5-CH_3-THFA:homocysteine transmethylase, two key enzymes most subject to control, particularly by L-methionine (e.g., Blakley, 1969; Silber and Mansouri, 1971). In the carrot system, some inhibition of the reductase was observed at low concentrations of L-methionine. Similar results have been observed in yeast (Combepine *et al.*, 1971) but higher plant tissues such as pea cotyledons and root tips do not appear to control this enzyme through methionine inhibition (Cossins *et al.*, 1972). While the 5-CH_3-THFA:homocysteine transmethylase in carrot tissue slices was inhibited to a small degree at relatively high concentrations of L-methionine, other systems such as yeast (Lor and Cossins, 1972), *E. coli* (Dawes and Foster, 1971) and chicken liver slices (Silber and Mansouri, 1971) regulate this enzyme by repression. Some higher plant tissues such as pea cotyledons and root tips appear to employ

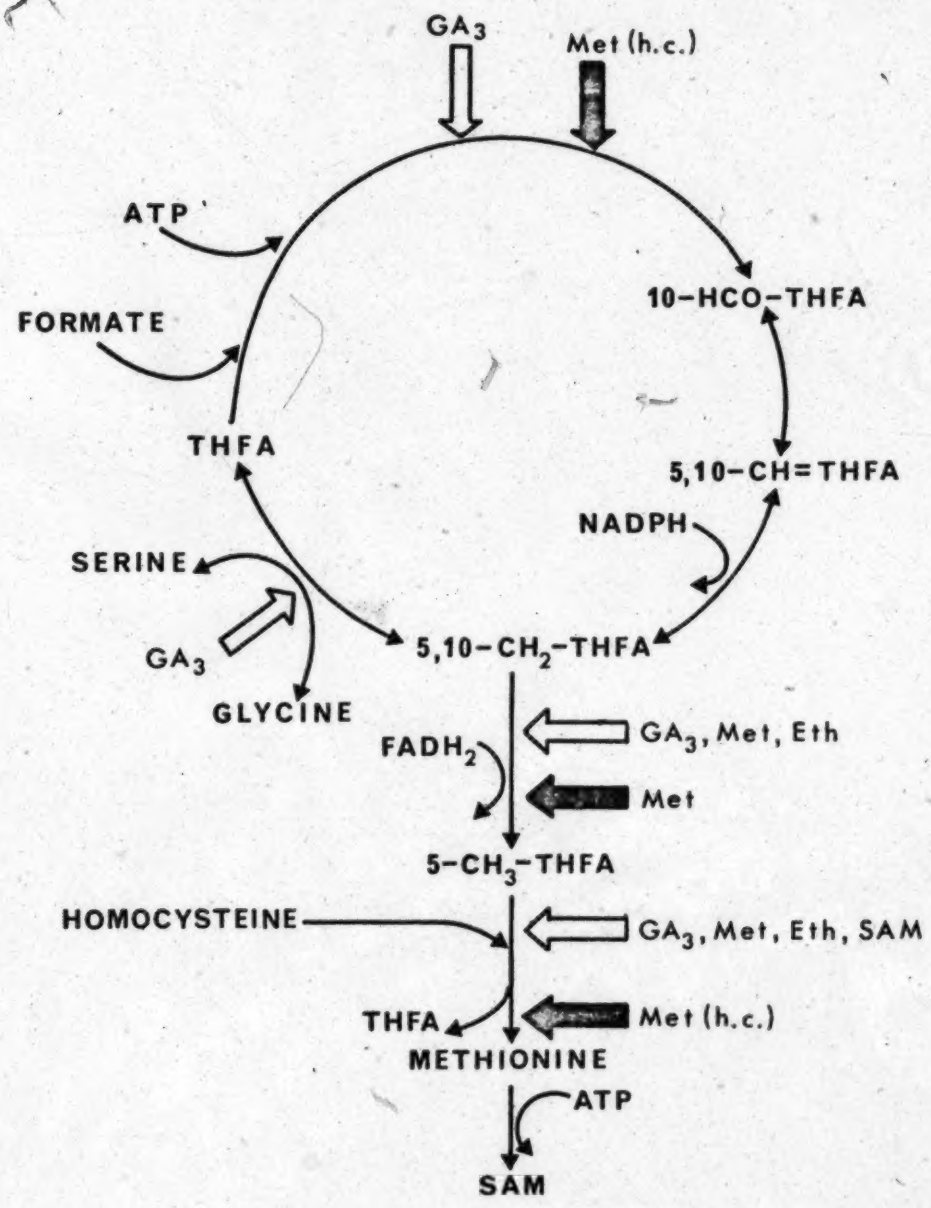


Fig. 10. *Regulation of methyltetrahydrofolate synthesis in aerated carrot tissue disks*

⇦, Increased specific enzyme activity (may be either: induction, activation or derepression); ⇦, inhibition of enzyme activity. h.c., high concentrations.

methionine inhibition as a control mechanism (Dodd and Cossins, 1970; Cossins *et al.*, 1972; Clandinin and Cossins, 1974). Such differences in the regulation of these two enzymes in different plant systems serve to emphasize the variability which exists in the mechanisms for control of methyl group synthesis.

Although the specific activity of serine hydroxymethyltransferase increased in carrot slices aerated in L-methionine, no other effects of methionine were noted. This amino acid is known to exert a regulatory role by causing repression of this enzyme in *E. coli* (Nakamura *et al.*, 1973) and by inhibition of its activity in yeast (Botsford and Parks, 1969). These regulatory mechanisms have not yet been found in higher plant tissues (Cossins *et al.*, 1972).

Partial inhibition of 10-HCO-THFA synthetase of yeast extracts by L-methionine has been observed recently (Lor and Cossins, 1972). A similar type of regulation could possibly operate in carrot tissue slices. However, until further clarification is made of possible competition between SAM synthetase and 10-HCO-THFA synthetase for available ATP in the assay, the apparent inhibition by L-methionine observed in the present investigation cannot really be adequately assessed (Table 21).

The apparent lack of strong repression or end-product inhibition by L-methionine raises the possibility that enzymes of C_1 metabolism in aerated carrot tissue disks may be alternatively controlled by intermediates along metabolic pathways leading away from the C_1 units produced directly by these enzymes. The role of GA_3 in enhancing the activity of the four enzymes studied is most likely an indirect effect but one worthy of further elucidation. Effects of nucleotides, ATP, glycine, serine and

SAM on these enzymes would certainly be worthy of close study as would the possible regulation of homocysteine biosynthesis in this system where the rates of methyl group synthesis and utilization must be very dynamic.

IV. Incorporation of [^{14}C]Formate by Aerated Carrot Tissue Slices

Feeding [^{14}C]formate to carrot tissue slices in an attempt to follow the flow of carbon through the C_1 pathway yielded much interesting information. Over 65-75% of the ^{14}C incorporated during the 1 hr feeding period was recovered as $^{14}CO_2$ (Table 22). This agrees favorably with earlier data of Cossins and Sinha (1965) where an extensive decarboxylation of formate by freshly-cut carrot slices was noted. In the present study, however, more label appeared in the organic fractions (Table 22) than was previously observed. Disks that had been aerated for 36 hr prior to incubation with [^{14}C]formate showed a greater capacity to incorporate ^{14}C into the organic acids and CO_2. These substantial incorporations are likely due to formate dehydrogenase activity (Cossins and Sinha, 1965) together with some refixation of $^{14}CO_2$, primarily into organic acids (Splittstoesser, 1966; Splittstoesser and Mazelis, 1967). A portion of the formate metabolized appeared to be incorporated into the one-carbon pool via 10-formylTHFA synthetase. Such ^{14}C would tend to enter the pools of serine and methionine (Cossins and Sinha, 1965) by pathways established some time ago in pigeon liver extracts by Kisliuk and Sakami (1954).

In these respects the findings of the present investigation are in close agreement with related research of other systems. For example, Tolbert (1955), in feeding [^{14}C]formate to darkened barley leaves, noted that the label appeared in the β-carbon of serine. In a similar study,

glutamate and serine, but not glycine were labelled after [^{14}C]formate feeding to darkened wheat leaves (Bowman and Rohringer, 1970). Likewise, Zemlyanukhin *et al.* (1972) found that [^{14}C]formate fed to corn leaves yielded $^{14}CO_2$ which was refixed into oxaloacetate and finally gave rise to labelled aspartate, glutamate as well as serine-3-^{14}C, the latter formed presumably from labelled methyleneTHFA.

In Lor and Cossins' (1972) work, yeast cells supplied [^{14}C]-formate synthesized labelled serine and cells cultured in an L-methionine medium, although showing a lower capacity to incorporate ^{14}C into other amino acids, converted more formate into serine and the adenosyl moiety of SAM. In the present investigation, carrot slices aerated in methionine also showed a greater capacity for incorporation of ^{14}C into SAM (6 M fraction, Table 23) but contrary to the behavior of yeast, a considerable amount of label appeared in methionine (Table 24, 25).

In a 3 hr feeding of [^{14}C]formate to freshly-cut carrot slices, Cossins and Sinha (1965) observed that 75% of the ^{14}C in the total free amino acid fraction was in serine and 16% was in methionine and its sulfoxide. In the present investigation, 67% of the label in the free amino acids was in serine (Table 25) for 36 hr aerated slices; 12.5% of the label was in glutamate and 8.9% in aspartate. A similar incorporation occurred in GA_3-aerated slices. On a total radioactivity basis, about the same number of counts may be observed in serine in methionine-treated slices as in the control, however, on a comparative basis, only 30.6% of the label incorporated during the feeding is as serine while the major portion (56.9%) is as methionine. Apparently the accumulation of methionine in the disks also acts as a trap for the radioactivity; subsequently, dilution of the specific activity resulted (Table 26).

GA_3 added to the medium of aerated carrot tissue slices, may be capable of increasing the methylation occurring in the tissue as suggested by King and Chapman (1973). This would then lead to increased amino acid acceptor capacity. Detailed analysis of free amino acids in GA_3-treated disks indicated their more extensive utilization in carrot slices given this treatment (Table 7). Residue analysis after [^{14}C]formate feeding indicated a more active incorporation of labelled amino acids into protein as evidenced by the higher percentage of label incorporated into residue as compared to the control (Table 23).

Detailed analysis of the protein amino acids after formate feeding of 36 hr aerated slices showed that the label appeared mostly in serine and aspartate in the control and GA_3-treated disks but in methionine-treated tissue, the label appeared mostly as glycine, aspartate and serine. Why methionine-treated slices showed so little label in protein amino acids and such a large accumulation of label in methionine is a question that warrants further study.

After [^{14}C]formate feeding, analysis of both free and protein amino acids showed a high specific activity occurring in methionine (Tables 26, 28) in all three treatments. The highest specific activity in free methionine was found in GA_3-treated slices indicating that a stimulation of the synthesis of C_1 units to meet increased catabolic demands outlined in Fig. 1 was induced by GA_3 treatment of this system.

V. Concluding Remarks

The results of the present investigation have shown that aerated carrot tissue slices possess the necessary enzymes for extensive C_1

metabolism. Increases in folate concentration, primarily 5-CH_3-THFA, occurring during the aeration period are undoubtedly related to an activation or synthesis of these enzymes.

Unlike the situation prevailing in microorganisms and animal tissues, exogenous methionine does not appear to play a vital role in regulating the biogenesis and utilization of methyl groups from the tetrahydrofolate pool. This amino acid is found in very low quantities in freshly sliced as well as aerated carrot disks, hence it is unlikely that the concentration in the tissue attains the levels where regulatory properties occur as observed in this study.

The large and rapid increases in folate derivatives found to occur during aeration, particularly in GA_3-treated disks, may be interrelated to enhanced methylation and nucleic acid synthesis which would result in routing methyl groups to meet these additional requirements. The enhanced enzymic activity observed could serve such a function but how and where GA_3 asserts its action remains to be investigated.

There is every indication from the evidence presented regarding the activation of enzymes of C_1 metabolism, the rapid depletion or accumulation of 5-CH_3-THFA and the incorporation of label from [^{14}C]formate into serine or methionine, that an active turnover of methyl groups occurs during aeration of these tissue slices. The rate of such turnover by the treatments used in this study could be determined by providing any of several labelled C_1 donors followed by the examination of radioactivity appearing in various cellular fractions.

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